THE UNIVERSITY OF ALBERTA

ASPECTS OF THE LIFE CYCLE OF *PROTOSTRONGYLUS* SPP., LUNGWORMS OF BIGHORN SHEEP (*OVIS CANADENSIS*)

by

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(C)

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ASPECTS OF THE LIFE CYCLE OF *PROTOSTRONGYLUS* SPP., LUNGWORMS OF BIGHORN SHEEP (*OVIS CANADENSIS*) submitted by JUDITH SAMSON in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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Supervisor

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Date June 28, 1984

ABSTRACT

The lungworms *Protostrongylus stilesi* and/or *Protostrongylus rushi* have been implicated as a major mortality factor in bighorn sheep (*Ovis canadensis canadensis*) die-offs occurring this century. Although basic aspects of the life cycle have been elucidated, mechanisms of transmission, the effects of *Protostrongylus* spp. on life history parameters and behavior of the snail intermediate host, and their effects on recruitment to bighorn sheep populations through lamb mortality are not clear.

First-stage *Protostrongylus* larvae infected the snail *Vallonia pulchella* mostly through penetration of the foot muscle, and less frequently by the oral route. The rate of development in *V. pulchella* was linearly related to temperature with a thermal threshold for development of 8°C and a thermal constant of 305 degree-days.

Lungworm infection did not affect fecundity or mortality of *V. pulchella*. As well, growth rates of offspring of infected snails were not impaired.

Some behavioral modifications were observed in infected snails. Their responses to stimuli such as heat, light or cover were the same as those of uninfected snails but infected snails seemed more active. However, it is difficult to assess whether or not those modifications would enhance transmission to the definitive host.

Each of three free-ranging bighorn lambs experimentally infected with 125-150 or 1000 infective *Protostrongylus*

larvae in 1982 and 1983 respectively had a marked increase in larval outputs following infection. The highest larval output (LPG = larvae per gram of dried feces) reached by experimental lambs before winter were 1090 LPG in 1982 and over 5000 LPG in 1983. Despite the abnormally high larval outputs, no clinical signs of pneumonia were observed among experimentally infected lambs were observed. A year after infection, larval shedding of the experimental lambs of 1982 that had become yearlings was not significantly different from that of naturally infected yearlings.

Several lines of evidence suggest that the prepatent period for *Protostrongylus* is less than 35 days. Transplacental transmission occurs at Ram Mountain as shown by the presence of *Protostrongylus* first-stage larvae in fecal samples of lambs approximately three weeks old.

Protostrongylus spp. possess several characteristics to enhance transmission to bighorn sheep: the use of two modes to infect their intermediate host, their ability to develop rapidly in snails at warm temperature to ensure presence of infective snails on winter ranges of bighorn sheep where density of the definitive host is high, no health damaging effects on the intermediate host to promote viable snail populations and transplacental transmission to increase chances of infecting bighorns where snail densities are low.

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I. INTRODUCTION

Bighorn sheep are considered one of North America's most valuable game animals because of their elusive nature and their pristine habitat. However, lung disorders, especially the lungworm-pneumonia complex (*Protostrongylus* spp infections complicated by bacterial or viral pneumonia) have been implicated as causal factors in many die-offs over the past 50 years (see reviews by Buechner, 1960; Forrester, 1971; and Hibler *et al.*, 1982). Bighorn mortalities attributable to the lungworm-pneumonia complex have reduced populations in the USA by up to 95% (Forrester and Senger, 1963). In Canada, sporadic all-age die-offs have been observed (Stelfox, 1971), and Stelfox suggested that populations of bighorn sheep seem to be naturally regulated by a lungworm pneumonia which prevents prolonged overpopulation with consequent forage destruction.

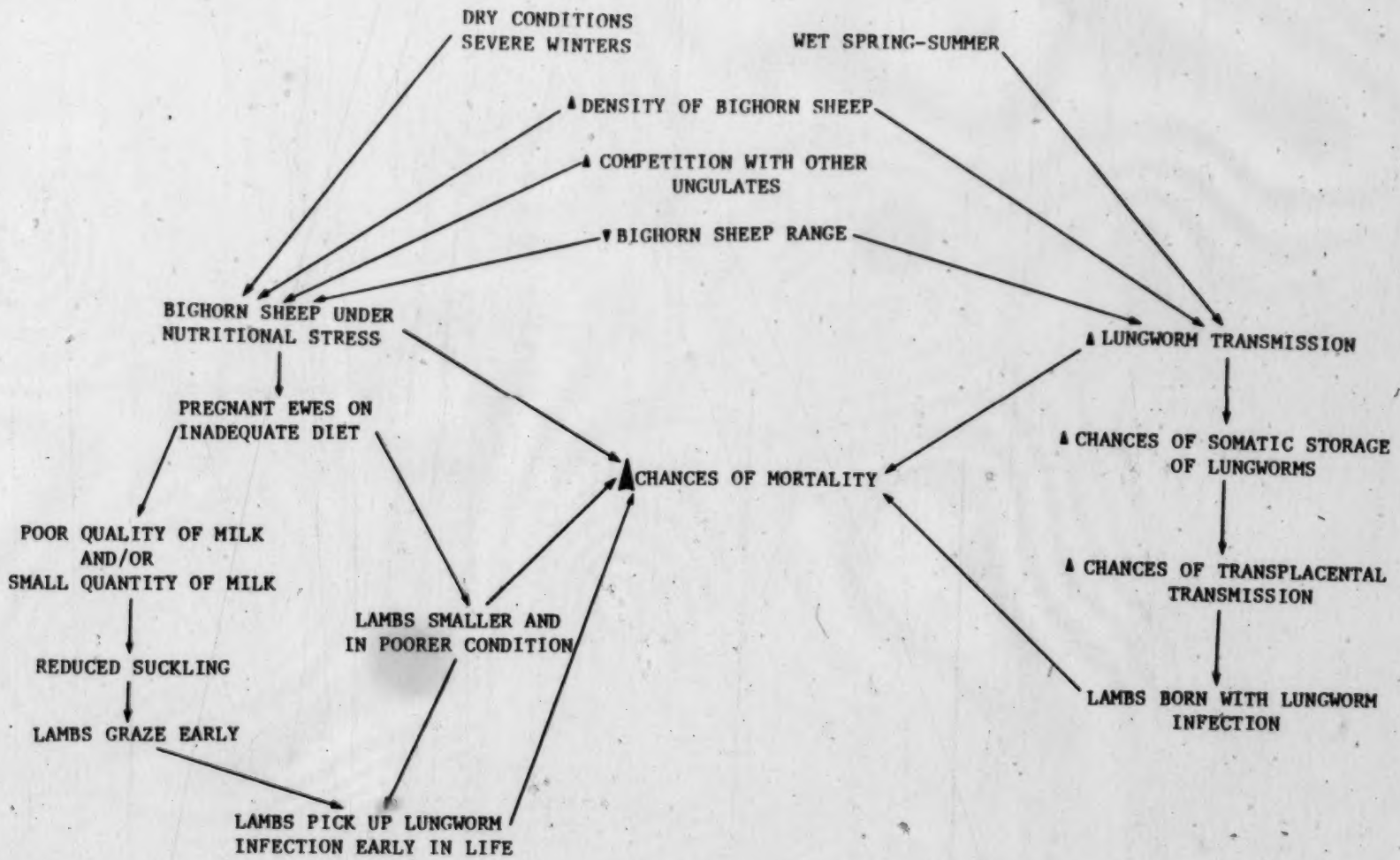
The lungworm-pneumonia complex also influences recruitment to bighorn sheep populations by acting as a major mortality factor among lambs. (see Buechner,1960 and Howe,1966 for mortality patterns; Spraker,1979 for pathology). Hibler *et al.* (1982) stated that pneumonia of bighorn lambs was responsible for 75-97% of the extensive recurrent lamb mortality during mid-summer and early fall at Pike's Peak, Colorado. Hibler *et al.*(1972,1974) and Spraker(1979) showed evidence of transplacental transmission of lungworms and believed that it was a causal factor in the mortality. In Canada, transplacental transmission is also

known to occur (Gates and Samuel,1977) and so is significant late summer-early fall mortality of lambs (Horejsi,1976).

Several factors have been or may be implicated in both the die-offs and the lamb mortality. Some of the possible inter-relationships between important factors are outlined in Figure 1. Mortality of bighorn lambs can be influenced by two types of factors: those that indirectly reduce their ability to resist diseases, such as nutritional stress on pregnant or lactating ewes (Hunter,1956; Butterworth and Blore,1969); and those that promote transmission of lungworms, such as high precipitation (Forrester,1969), a high density of sheep (see review in Buechner,1960), and poor range conditions (Demarchi and Demarchi,1967). Lamb mortality seems to occur when there is some combination of these factors acting at once (Horejsi,1976).

However, little attention has been paid to what may well be the most influential determinant of the outcome of a parasite-host encounter, the immunocompetency of the host. Where young animals are exposed to parasitic infections, it is important to consider both the ability of the animal to respond while immunologically immature, and the significance of early exposure on subsequent immunologic development. Domestic animals under six months of age may or may not have impaired responses to immunization with some sensitizing parasitic infections but not to others. Manton *et al.*(1962) demonstrated that Blackface lambs were not able to mount an effective immune response to *Haemonchus contortus* until they

Figure 1. Factors that may be implicated in bighorn lamb mortality.


DRY CONDITIONS
SEVERE WINTERS
WET SPRING-SUMMER
▲ DENSITY OF BIGHORN SHEEP
▲ COMPETITION WITH OTHER UNGULATES
▼ BIGHORN SHEEP RANGE
BIGHORN SHEEP UNDER NUTRITIONAL STRESS
▲ LUNGWORM TRANSMISSION
PREGNANT EWES ON INADEQUATE DIET
▲ CHANCES OF SOMATIC STORAGE OF LUNGWORMS
▲ CHANCES OF MORTALITY
POOR QUALITY OF MILK AND/OR SMALL QUANTITY OF MILK
▲ CHANCES OF TRANSPLACENTAL TRANSMISSION
LAMBS SMALLER AND IN POORER CONDITION
REDUCED SUCKLING
LAMBS BORN WITH LUNGWORM INFECTION
LAMBS GRAZE EARLY
LAMBS PICK UP LUNGWORM INFECTION EARLY IN LIFE

were four to six months old. Ross (1970) has observed that the ability of domestic lambs to resist *Trichostrongylus* infections was almost nil until the age of five months, but greatly improved by the seventh month. However, Halliday (1978) reviewed some of the literature and believed that domestic lambs two to four months of age become as resistant, and respond as vigorously as adults to a variety of infections.

Early infection can also result in immune tolerance-a permanent impairment of resistance (Kassai and Aitken, 1967), although persistence of the antigen may be necessary for the maintenance of tolerance (Jarrett *et al.*,1968; Jarrett,1971). Gibson (1952) demonstrated that acquired resistance to parasites may be depressed by massive early infection. He experimentally infected several one month old lambs with single doses of 10000, 15000, and 20000 *Trichostrongylus axei* larvae and observed respectively, strong resistance, no effect, and decreased resistance when challenged again. Thus, the role and mechanisms of immunologic tolerance remain unclear although there seems to exist a reduced responsiveness to parasitic infections when these occur early in life.

These studies suggest that for *Protostrongylus* in bighorn sheep, prenatal lungworm infection, or early post-natal infection, could result in impairment of the immune response by the induction of tolerance. This phenomenon is not necessarily detrimental to the host but

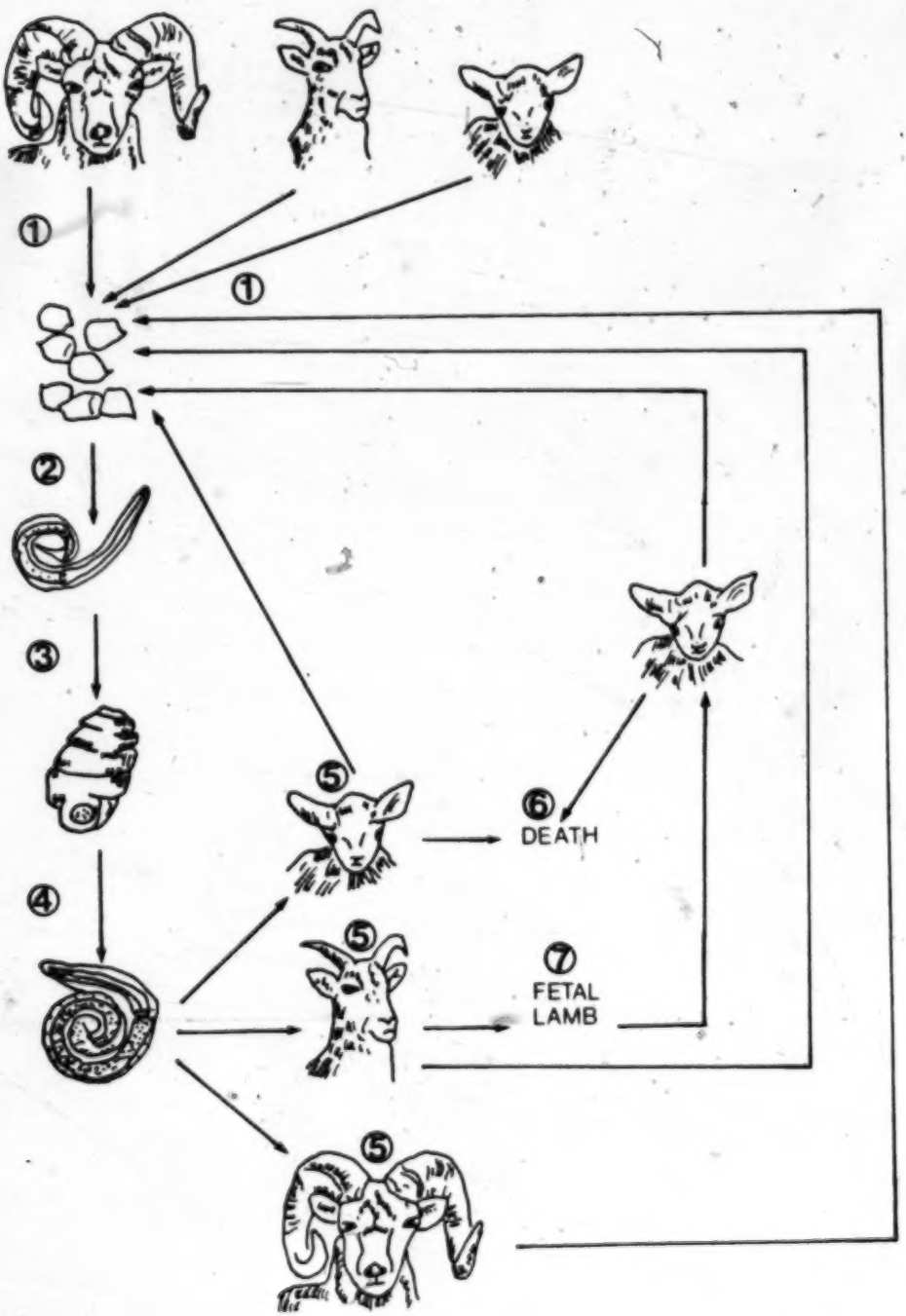
may reflect an evolutionary equilibrium between the immune response and the parasite population whereby the parasite may carry on efficiently its functions with a minimal pathologic insult to the host (Damian,1964). However, when this equilibrium is broken, perhaps by extensive early infection, the result may be fatal to the host.

The lungworm-pneumonia complex in bighorn sheep is characterized by an acute, often fatal course in lambs followed by a chronic course in adults that have survived the acute phase (Howe,1966). According to current concepts, as expressed by Forrester (1971) and Hibler *et al.* (1982), the lungworms *Protostrongylus stilesi* Dikmans, 1931 and *Protostrongylus rushi* Dikmans, 1931 act as predisposing agents which condition the lungs for pneumonia through secondary microbial infection, primarily by bacteria of the genera *Pasteurella*, *Corynebacterium*, *Streptococcus*, and *Neisseria* (Hibler *et al.*,1982) or by myxoviruses (Howe,1966).

Since one of the major etiological factors in the lungworm-pneumonia complex is the lungworm, it is important to know its life cycle, especially the mechanisms of transmission. The life cycle of protostrongylid lungworms, which involves a molluscan intermediate host, is shown in Figure 2.

After being fertilized in the lungs (*P. stilesi* in the parenchyma and *P. rushi* in the bronchi), female adult worms lay eggs which hatch into first-stage larvae (L_1s). These

Figure 2. Life cycle of Protostrongylus stilesi in bighorn sheep. (Modified from Hibler et al.,1982; see text for explanation).



larvae break out into the alveoli (*P. stilesi*), migrate up the respiratory passages, are swallowed and passed in the feces (1 in Fig.2). Larvae on the pasture (2) can survive at least one year (Lange,1973) and are very resistant to extreme environmental conditions such as temperature and humidity (Honess and Frost,1942; Couey,1950). First-stage larvae invade or are eaten by certain terrestrial snails (3). Snails that serve as intermediate hosts for *Protostrongylus* larvae are mostly small and inconspicuous members of the genera *Vallonia*, *Pupilla*, *Gastrocopta*, *Pupoides*, *Vertigo*, and *Euconulus* (reviewed by Buechner,1960; Forrester,1971).

Very few protostrongylid lungworms have been examined for their mode of entry into their intermediate host. Hobmaier and Hobmaier (1934) claimed that lungworm larvae ingested by snails do not survive and only those that have penetrated the foot become infective. However, Kralka (1983) has observed that *Protostrongylus boughtoni*, a parasite of snowshoe hare, can get in the snail *Vallonia pulchella* only via ingestion. Platt and Samuel (1984) reported that *Parelaphostrongylus odocoilei* larvae use either an active mode of entry (penetration of the foot tissue) or a passive mode (ingestion) depending on the species of their gastropod intermediate host. It is still unclear how *P. stilesi* and *P. rushi* gain access to snails.

Once in the intermediate host, the larvae molt twice to reach the infective third-stage (L_3) (4). Hibler *et al.*

(1982) stated that development of protostrongylids of bighorn sheep to the L_3s requires 45 to 60 days, depending upon environmental conditions. As is normal in poikilotherms, the development of nematodes that use snails as intermediate hosts is temperature-dependent, with the quickest development at high temperatures. However the details of temperature dependence such as the rate of lungworm development at specific temperatures, which could lead to the interpretation of biological data on infected snails in natural conditions, are unknown.

To complete the life cycle, infective larvae must be eaten by a bighorn (5). Most consider "accidental ingestion" of snails containing infective larvae as the mechanism by which sheep acquire lungworms. However, the apparent efficiency of transmission reflected in the prevalence of infection in Rocky Mountain bighorn sheep (100%:Forrester,1971) and in snails infected (5-10%:Latson,1977), suggests that the parasite may alter its intermediate host's behavior to enhance transmission.

Once the L_3s are in the gastro-intestinal tract of the definitive host, they migrate via an unknown route to the lungs and develop to the adult stage. The prepatent period is not known. As indicated earlier, lungworms may be acquired transplacentally (7) and if this transplacental infection (or other early infection) is severe, the lamb may die (6). Lambs with extensive lungworm infections have frequent paroxysms of coughing, shaggy hair coats, are small

in size and light in body weight compared to healthy lambs, and seldom frolic (Spraker, 1979).

As is obvious from the above discussion, many aspects of the mechanisms of lungworm transmission and their effects on both the intermediate and definitive hosts are poorly understood. In particular, nothing is known about potential effects of larval *Protostrongylus* on the survival, reproduction, or behavior of the snail hosts. Therefore, this thesis examined aspects of the life cycle of the lungworms in a laboratory-infected intermediate host snail, *Vallonia pulchella*, and infectivity in free-ranging bighorn lambs. Specific objectives of the study were:

1) To clarify two aspects of the life cycle of *Protostrongylus* species in the intermediate host, namely a) the mode(s) of entry used by first-stage *Protostrongylus* larvae to infect *V. pulchella*, and b) the effect of temperature on the rate of development of *Protostrongylus* larvae in *V. pulchella*.

2) To determine the effects of *Protostrongylus* larvae on three life history parameters of *V. pulchella*: a) the rate of egg production, b) the rate of mortality, and c) the growth rate of offspring of infected snails.

3) To determine whether or not lungworms alter the behavior of *V. pulchella*.

4) To determine whether or not transplacental transmission occurs in the bighorn sheep population at Ram Mountain.

5) To determine whether or not extensive early exposure to *Protostrongylus* lungworms can induce pneumonia in bighorn lambs.

II. MATERIALS AND METHODS

Since larval stages of *Protostrongylus* species (namely *Protostrongylus stilesi* and *P. rushi*) found in bighorn sheep cannot be distinguished, they will be referred to throughout the rest of this thesis as "*Protostrongylus*". When reference to other species of *Protostrongylus* is meant, "species of *Protostrongylus*" will be used.

Vallonia pulchella snails used in this study were collected from a lungworm-free colony found on the campus of the University of Alberta, Edmonton, or were laboratory-reared descendents of members of that colony. This colony is an introduced one, found associated with an introduced Manitoba maple tree (*Acer negrundo*) on campus. Although *V. pulchella* has a wide distribution in North America (east of the Rocky Mountains, from southern Canada to Missouri and Kentucky: Burch, 1962), it has never been reported on bighorn sheep ranges in Alberta. However *V. pulchella* has been found naturally infected with protostrongylid lungworms in Colorado (Pillmore, 1955). Since this snail is a natural intermediate host for *Protostrongylus*, I considered it a suitable host.

Snails were maintained in a terrarium containing soil and leaf litter collected from the same site on campus. Distilled water was sprayed onto the leaf litter twice a week; and once a month a few grams of $CaCO_3$ was added to the water. The snails were continuously fed leaf litter and lettuce.

Snails were infected by placing about 50 of them in a petri dish (7.8cm diam.), lined with filter paper discs (Whatman #1) onto which was poured tap water containing approximately 10,000 first-stage *Protostrongylus* larvae, recovered from bighorn sheep feces using a modification of the Baermann technique (Samuel and Gray, 1982).

MODE OF ENTRY

A total of 56 specimens of *V. pulchella* was exposed for a two-hour period, after which seven snails were fixed immediately in Bouin's fixative and the remaining snails were returned to a finger bowl containing sterilized soil and leaf litter. Groups of 5-7 of these snails were fixed at 3, 6, 9, 12, 24, 48, and 72 hours post-exposure. The snails were left in the Bouin's solution for 9 days and then dehydrated in a graded series of alcohol, embedded in paraffin, serially sectioned at 7um, stained with Harris' hematoxylin and counterstained in eosin. All sections from each snail were examined using a compound microscope for detection of larvae. The number and locations of all larvae were recorded.

EFFECT OF TEMPERATURE ON DEVELOPMENT OF *PROTOSTRONGYLUS* IN *V. PULCHELLA*

Seventeen colonies, each containing 30 infected snails, were used in the experiment. Four colonies were kept at each of 30°C, 25°C, 20°C, 15°C, and one at 10°C. To monitor

larval development, snails were placed on a wet slide and allowed to move freely. The slide was inverted under a dissecting microscope to allow examination of the snail feet where the larvae occur. The number of snails in each colony containing third-stage larvae, identified by their dark brown cuticle (Pillmore, 1955), was recorded. Based on preliminary results, snails in the colonies kept at 30°C, 25°C, and 20°C were checked every second day, those at 15°C every fourth day and the one at 10°C, weekly. In another experiment, designed to verify whether or not rate of development of larvae in the snail host would be affected by a change of temperature, groups of 10 snails were transferred to 25°C after 18, 32, 46 or 50 days at 10°C.

The date on which at least half of the snails in each colony contained a third larval stage was determined. This date was used to estimate T_{50} ("time required for 50% of the larvae to reach a particular developmental stage"(Tokeson and Holmes, 1982)). The constants of a linear regression of the rate of development ($1/T_{50}$) on temperature were used to measure the thermal threshold (the temperature below which no measurable development occurs, calculated as -a/b) and the thermal constant (the number of degree-days required to complete development, calculated as 1/b) (Campbell *et al.*, 1974).

EFFECT OF *PROTOSTRONGYLUS* INFECTION ON FECUNDITY, MORTALITY AND GROWTH RATE OF *V. PULCHELLA*

Twenty-five colonies of 30 snails each were used in the experiment. When the experiment started, four colonies of infected snails and four colonies of uninfected snails were kept at each of 30°C, 25°C and 15°C, and one colony of uninfected snails at 10°C. Every four days, the number of snail eggs present in a colony was recorded and the eggs removed from the colony. At the same time, all snails within the colony were examined under a dissecting scope in order to check their viability. The number of dead snails was recorded.

The median test (Siegel, 1956) was used to compare the number of eggs per snail-day and the number of dead snails between infected and uninfected colonies, before and after the first larval moult (the first moult occurring at day 8 at 30°C, day 12 at 25°C, and day 20 at 15°C). Pillmore (1955) showed that maximum size of *Protostrongylus* larvae is attained at the time of the first moult; thus the greatest energetic demand, that could possibly impair some of the host's physiological functions, should be made on the host at that time. For each of the two parameters, egg-laying and mortality, the median test was performed by comparing each of the four values of infected and uninfected colonies with the median for each day. Total numbers before (and after) the first moult were used in the test.

Determination of growth rates of young snails born to infected and uninfected *V. pulchella* was done by removing eggs from colonies of infected and uninfected snails and placing them in finger bowls lined with soil and leaf litter and kept at three different temperatures, 10°C, 15°C and 25°C. At each of the three temperatures, there were two colonies of 50 eggs each, one colony from infected parents and the other one from uninfected parents. As soon as the young snails were large enough to be readily visible (at least 0.5 mm), half of them were measured weekly (only half were measured to reduce mortality, because the young snails have their shells easily broken from handling). Measurements of the diameter of shells were done using a calibrated ocular micrometer in a compound microscope.

EFFECT OF *PROTOSTRONGYLUS* ON BEHAVIOR OF *V. PULCHELLA*

Temperature gradient

The responses of infected and uninfected snails were tested in a temperature gradient between 30±2°C and 10±3°C established in a rectangular metal dish (30cm X 19cm), lined with wet filter paper, placed half on a platform heater and half on a bowl of ice. Three experiments were conducted, each using two groups of 15 uninfected snails and two groups of 15 infected snails. Each experiment was conducted in the light for 75 minutes, the maximum time for which the temperature gradient could be controlled. Every 15 minutes, the position of snails (in the warm or in the cool side) was

recorded. For experiment one, snails were initially placed in the middle of the heat gradient, for experiment two, the snails were placed in the cool side of the dish, and for experiment three, the snails were started in the warm side of the dish. In experiment two and three, a line was drawn where all snails were started. If they moved from that line toward the cool area, the position was recorded as being in the cool side. If they moved from the line toward the warmer area, the position was recorded as being in the warm side even though the snails had not gone beyond a line that was drawn across the middle of the dish. A chi-square test (Siegel,1956) was used to determine whether or not total numbers of snails that moved into the warm side differed from random and whether or not infected snails tended to get "trapped" more (or less) frequently than uninfected ones. (If a snail enters a favorable area, will it tend to stay there -"be trapped"- whereas if it enters an unfavorable area, will it tend to leave, thus "not be trapped"?)

Reaction to light

The preferences of uninfected and infected snails for light or dark conditions were tested in a petri dish (9cm diam) half-covered with two layers of dark green plastic material. A heatless lamp was used as a source of light. The bottom part of the petri dish was lined with wet filter paper on which snails were allowed to crawl. Four groups of ten uninfected snails each and four groups of ten infected

snails each were started in the dark side. Every 15 minutes for two hours, their positions (in the dark or in the light side) were recorded. This procedure was repeated, placing the snails in the light side. Differences in total numbers of infected and uninfected snails in the light or in the dark were tested using a sign test (Siegel, 1956).

Rate of activity

The rate of activity of infected and uninfected snails was examined in light conditions using a heatless lamp. Each of the twenty infected snails and twenty uninfected snails were put singly in a petri dish (9cm diam) lined with wet filter paper. They were allowed to crawl for 70 minutes. On the lid of each petri dish a line was traced to follow the movements of the snails. Every five minutes, the distance travelled by snails was recorded by measuring the length of the line traced. The total distance in millimeters that each of the twenty infected snails travelled was tested against the total distance that each of the twenty uninfected snails covered using a Mann-Whitney-U test (Siegel, 1956).

Attraction to leaf litter

To determine whether or not infected snails would seek vegetation cover more or less than uninfected snails, ten groups of ten uninfected snails and ten groups of ten infected snails were placed in 9cm petri dishes lined with filter paper and half covered with leaf litter material.

Snails were started in the half not covered with leaf litter. Their positions (in or out of leaf litter material) were recorded every 15 minutes for 90 minutes. A Mann-Whitney-U test was used to test whether or not numbers of infected and uninfected snails per colony found in the leaf litter after 90 minutes differed and a chi-square test was performed to test whether or not total numbers of infected and uninfected snails in the leaf litter differed.

EXPERIMENTAL INFECTION OF BIGHORN LAMBS

Description of the study area

Ram Mountain (52° 25'North, 115° 45'West) is the southernmost peak in the Brazeau range and is bounded on the north by the North Saskatchewan River. The range, with its valleys oriented northwest to southeast, is composed of a varied terrain of bare rock summits, talus and wooded slopes, low relief alpine tundra and rugged escarpments. The extreme elevations of the range vary between 1082m and 2173m with treeline at about 1500m. Weather conditions on the mountain are extremely variable. For more detail of the area, see Johnson (1975).

The flora above timberline is comprised mostly of low growing forms of plants such as saxifrages (*Saxifraga* spp.) and moss campion (*Silene acaulis*). In the alpine meadows, sedge (*Carex nardina*) and wheat grass (*Agropyron latiglume*) are abundant. The subalpine forest found at or below timberline is composed of white spruce (*Picea glauca* vars.

porsildii and *albertiana*), subalpine fir (*Abies lasiocarpa*) and lodgepole pine (*Pinus contorta* var *latifolia*) (Johnson, 1975). The most frequently observed mammals on the mountain during the summer are mule deer, moose, marmots, ground and red squirrels and pikas, in addition to bighorn sheep.

Description of the herd

At the end of the summer of 1982, the total population of bighorns on Ram Mountain was 111 (Jorgenson and Wishart,1982) . It is a young herd with 65% of the population less than 4 years of age. The sex ratio (males:females) was 1:1.1. Eighty-one percent of the reproductively active ewes lactated, and 26 lambs were born. Neonate mortality was approximately 16%. The 1982 lamb crop had a 1:1.6 male to female sex ratio. Generally, according to Geist's (1971) criteria, the Ram Mountain herd is one of high quality because of its high productivity, high lamb survival, low life expectancy, and fast growth rates.

Experimental protocol

Lambs required for experimental infection were captured along with other sheep in a corral trap using salt as a bait. Upon capture, standard body and weight measurements (for use by Alberta Fish and Wildlife) were taken and identification tags were affixed to the ears.

Seven lambs were caught on June 10,1982 and three of them were given 125-150 infective larvae (L_3s) of

Protostrongylus spp. upon capture. On August 21,1982, each of two additional lambs was infected with the same dose. The following year, each of three lambs was infected with 1000 L_3s on June 17 (n=2) and June 22 (n=1). The L_3s used were recovered from experimentally infected snails, *Vallonia pulchella*. The foot of each snail containing larvae was severed and put in a saline solution. The L_3s in this solution were fed orally to each lamb using a syringe with a plastic tube fixed at its end. The tube was inserted directly into the back of the mouth of the lamb to ensure that the solution containing the infective larvae was swallowed completely. A petri dish was put under the lamb's mouth in the event that some of the solution was coughed up. When that happened, the solution in the petri dish was checked for the presence of larvae under the microscope. If present, the procedure was repeated. The syringe was always rinsed and the lamb fed the rinse to ensure that all larvae were given to the individual lamb. These experimentally infected lambs will be referred to in the rest of this thesis as "experimental lambs".

The free-ranging lambs not given experimental infections served as control lambs. In 1982, all (n=21) lambs were captured and submitted to the same treatment as the experimental lambs except for the absence of larvae in the saline. In 1983, almost all lambs were captured, but not given saline, since this treatment did not have an effect on the lambs in 1982. These lambs are referred to in the rest

of this thesis as "naturally infected" lambs.

All experimental lambs were radio-collared and their movements were monitored throughout the summer. Fecal samples were collected as often as possible by locating the lamb with the radio-receiver, following it with a variable-power spotting scope (Bushnell 45X) until it defecated, having an observer mark the spot and keep it under constant observation, directing a second person to the site until the fresh feces were found. The behavior (play and resting time) of experimental lambs and naturally infected lambs was compared subjectively.

After October, snowfalls prevented easy access to the mountain; in November 1982 and January 1983, surveys were conducted by helicopter, and additional fecal samples were collected from the experimental lambs and other members of the herd.

III. RESULTS

MODE OF ENTRY

In the histological sections, first-stage *Protostrongylus* larvae were found in or penetrating the epithelium of the foot (Fig.3a,b), the mantle (Fig.3b), the mucosa of the intestine (Fig.3c) or the esophagus, and in the intestinal lumen of *Vallonia pulchella*. Many of the larvae were also found in the pharyngeal muscles (Fig.3d). The locations of these larvae indicate that first-stage *Protostrongylus* larvae are capable of infecting *Vallonia pulchella* by penetration of the surface epithelia, or by ingestion.

From zero to six hours post-exposure, approximately 22% of the larvae were found in each of the pharyngeal muscles and the alimentary tract, about 45% in the foot and about 10% in the mantle (Table 1). About two thirds of the larvae in the alimentary system were in the tissues of the esophagus or the intestine, and the other third in the lumen. Larvae in the gut wall were assumed to have penetrated after having been ingested; however, as indicated below, larvae in the intestinal lumen cannot be assumed to be destined to penetrate. Since the pharyngeal muscles lie close to both the surface of the proboscis, which is in contact with the substrate when the snail is crawling, and the pharynx, larvae in the pharyngeal muscles can be interpreted as having penetrated the tissues of the anterior

Figure 3. First-stage *Protostrongylus* larvae (arrows) in various tissues of *Vallonia pulchella*

a)Larva outside the epithelium of a fold of the foot muscle (penetrating in another section), and a larva already in the foot muscle (F).

b)Larvae in the mantle (M), and in the foot muscle.

c)Larva penetrating the intestinal (INT) wall.

d)Larva in the pharyngeal muscles (PH).

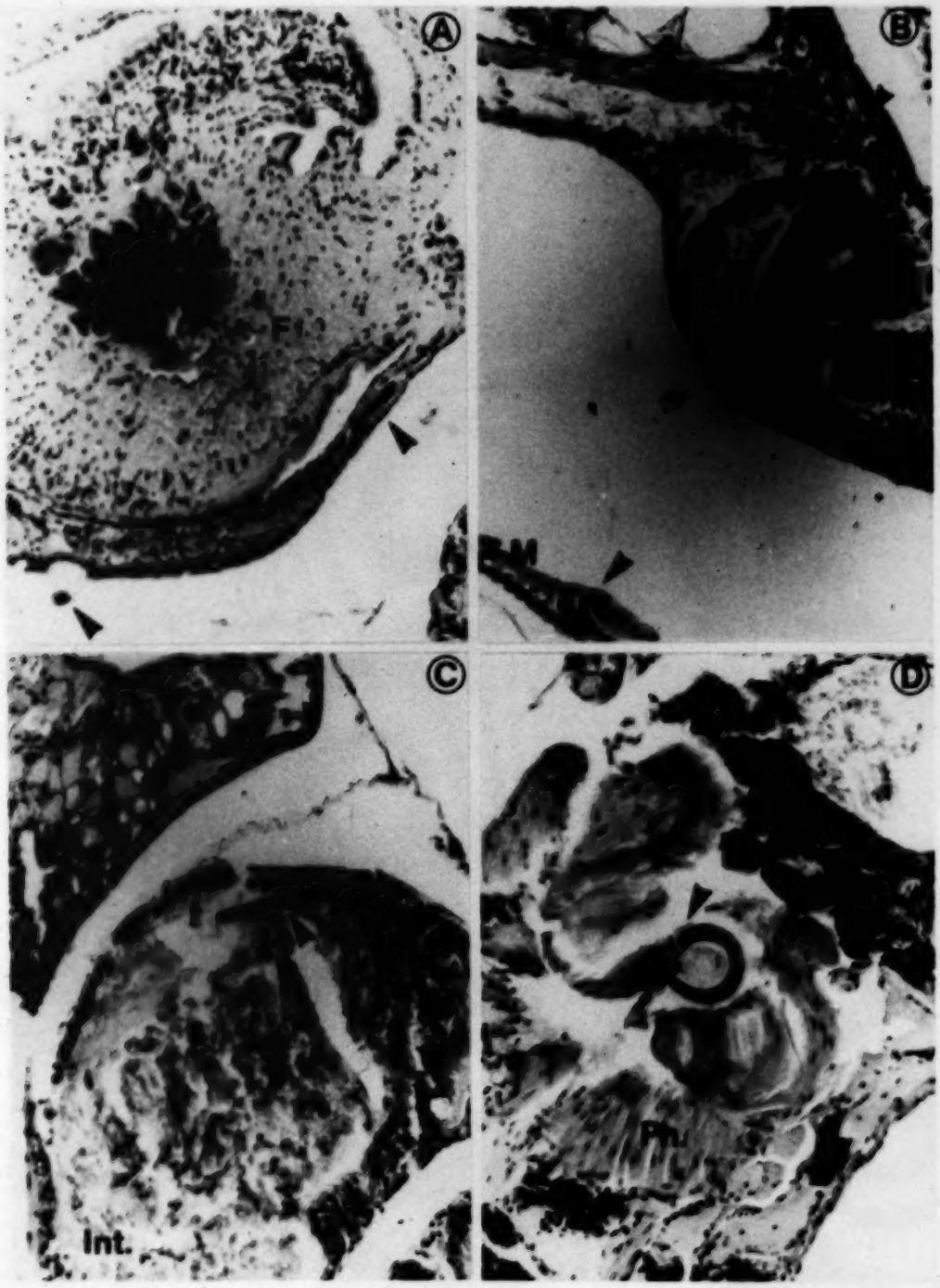

A
B
C
D
Int.
Ph

Table 1. . Location of Protostrongylus larvae in V. pulchella at different time intervals after exposure.

Time after 2hr exposure	no. snails	total L_1s	$\bar{X}$ no. larvae /snail ± SD	Mantle n	Mantle %	Foot n	Foot %	Pharyngeal muscles n	Pharyngeal muscles %	Alimentary tract n	Alimentary tract %	Others n	Others %
0	4	21	5.3 ± 1.0	2(0)*	10	5	24	7	33	4+3**	34	0+0***	0
3	4	19	4.8 ± 1.5	1(0)	5	10	53	5	26	3+0	16	0+0	0
6	4	18	4.5 ± 1.7	3(0)	17	10	56	1	6	2+1	17	0+1	5
9	7	53	7.6 ± 2.1	12(3)	23	19	36	3	6	14+3	32	2+0	4
12	6	58	9.7 ± 3.7	17(9)	29	24	41	5	9	8+1	16	3+0	5
24	4	31	7.8 ± 1.5	10(0)	32	16	52	2	6	2+1	10	0+0	0
48	5	41	8.2 ± 1.6	16(7)	39	14	34	5	12	2+2	10	2+0	5
72	7	84	12.0 ± 2.6	28(12)	33	39	46	3	4	3+5	10	3+2	6

*Number of larvae penetrating

**Number of larvae in gut wall and number of larvae in the intestinal lumen

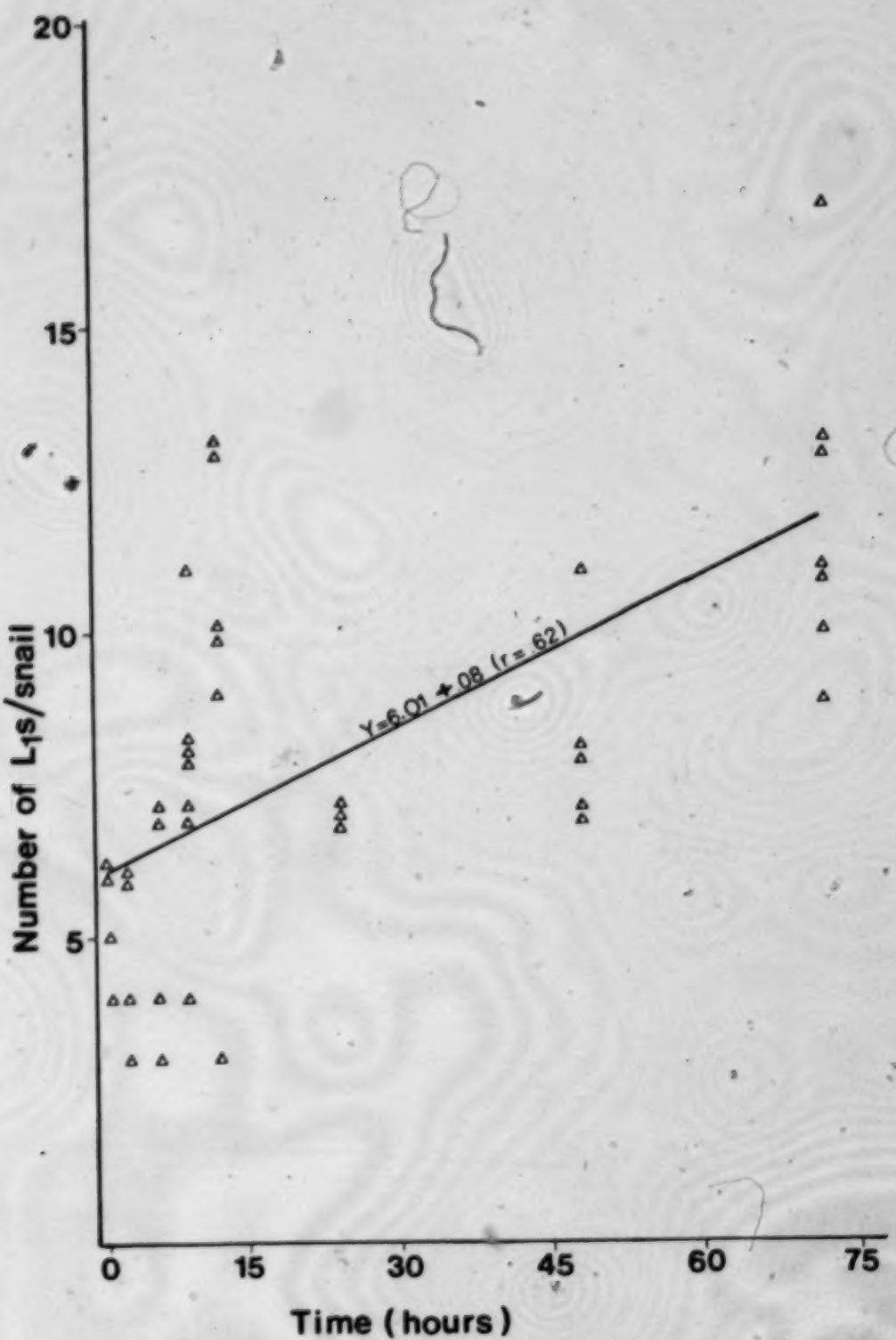
***Number of larvae in tissues and number of larvae in section but not in snail tissue

part of the foot or proboscis during exploratory movements of the latter or can be interpreted as having been ingested and migrated out of the esophagus. Therefore, it is not clear how the larvae in these muscles entered the snails, so that these larvae and those in the intestinal lumen have been disregarded. Of the remaining larvae 23% entered via the oral route whereas 77% penetrated directly.

Although the snails had been removed from the source of infection, the mean number of larvae per snail increased with time after the two-hour exposure ($r=.620$, $p<.025$)(Fig.4). The greater number of larvae per snail at the end of the experiment than at the beginning and the presence of larvae in the act of penetration through 72h post-exposure (Table 1) suggest that snails were continuously reinfected despite the removal of the source of infection.

Not only were there more larvae per snail, but locations of larvae differed. The number of larvae in the mantle augmented with time ($r=.557$, $p<.01$) while the number of larvae in the pharyngeal muscles, in the foot, and in the gut did not change significantly with time (pharyngeal muscles: $r=.230$, $p>.05$; foot: $r=.134$, $p>.05$; intestinal wall: $r=.232$, $p>.05$; intestinal lumen: $r=.243$, $p>.05$). Presence of larvae in sites where direct penetration could not occur (e.g., tissues nearby the intestine) indicated that migration of larvae to different tissues took place. Larvae in tissues did not show any signs of degeneration

Figure 4. Influence of time after a two-hour exposure on the number of Protostrongylus larvae per snail.



such as broken cuticle or cell autolysis.

EFFECT OF TEMPERATURE ON DEVELOPMENT OF *PROTOSTRONGYLUS* IN *V. PULCHELLA*

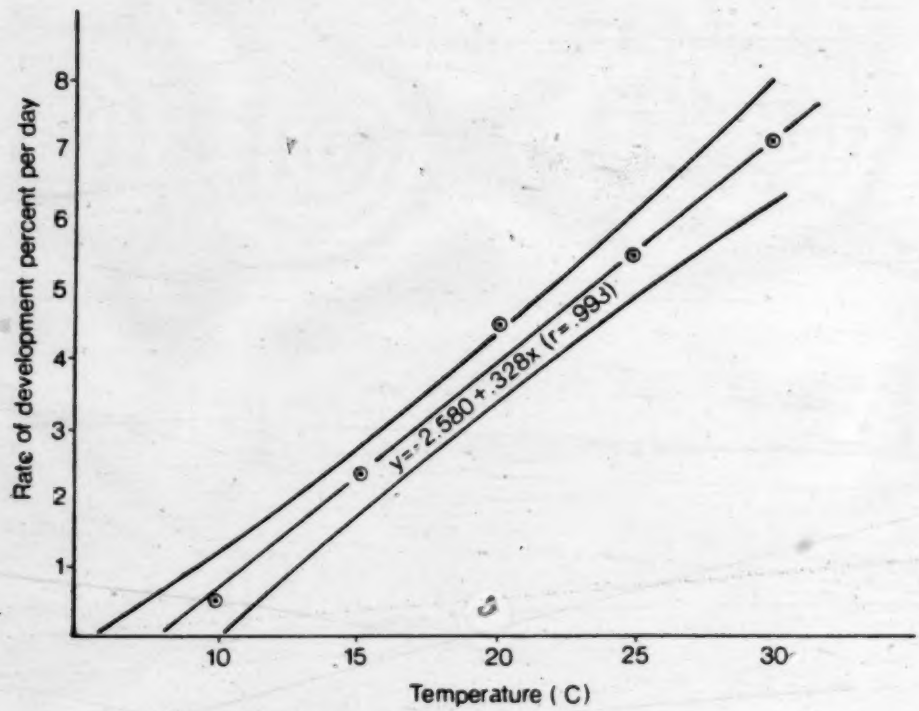
Time of development of *Protostrongylus* larvae in the intermediate host *V. pulchella*, within a suitable temperature range (10°C-30°C), was inversely correlated to temperature: at 30°C, T_{50} to the infective stage was approximately 2 weeks, whereas at 10°C, T_{50} was more than 10 times as long (Table 2). The rate of development to the infective stage was linearly related to temperature ($r=.993$, $p<.001$); the regression accounted for over 98% of the variance (Fig.5). The thermal threshold for development was 8°C and the thermal constant was 305 degree-days.

Infected snails kept at 10°C for 18, 32, 46 and 50 days took 15, 13, 11, and 11 days, respectively, to produce L_3s when incubated at 25°C. They developed significantly faster than expected (95%, 92%, 88%, and 91% of expected time, respectively) (combined probability $p<.005$). The percentages were tested and their probabilities combined as outlined in Sokal and Rohlf (1969). The expected times were obtained as follows: 2°C (10°C-8°C, the thermal threshold), was multiplied by the number of days spent at 10°C, the resulting number was substracted from 305, the thermal constant in degree-days, to give the number of degree-days expected. The actual number of degree-days, being the number of days required at 25°C for larvae to become infective

Table 2. Length of development (days) of *Protostrongylus* larvae in *Vallonia pulchella* at five constant temperatures.

Developmental period (to L_3)	Temperature 30°C	25°C	20°C	15°C	10°C
Mean	14.5	18.5	23.0	44.3	181.0
S.D	1.0	1.0	1.1	1.5	-
Mean no. of L_3/snail	9	12	7	8	13

Figure 5. Effect of temperature on the rate of development to the third larval stage of Protostrongylus in Vallonia pulchella. The regression line is enclosed in the 95% confidence intervals.



multiplied by 17 (25°C-8°C), was divided by the number of degree-days expected to give the percentage of the expected time. Thus, development was retarded at lower temperatures but this retardation did not inhibit, and may even have enhanced, the developmental capacity of larvae at later warmer temperatures (25°C).

EFFECT OF *PROTOSTRONGYLUS* INFECTION ON FECUNDITY, MORTALITY AND GROWTH RATE OF *V.PULCHELLA*

The number of eggs laid by either the infected or uninfected snails was greater at the beginning of the experiment than after one month suggesting that the snails might have become reproductively exhausted (Fig.6). In addition, fecundity of all snails was less at 15°C than at 25°C or 30°C ($p<.05$), and at 10°C, snails did not reproduce, indicating that low temperatures inhibit reproduction. The effect of *Protostrongylus* infection on fecundity of *V. pulchella* was nil; there was no significant difference at any temperature in egg-laying between infected and uninfected snails.

Protostrongylus infection did not cause a greater mortality of *V. pulchella* than in non-parasitized snails since the number of dead infected snails did not differ significantly from the number of dead uninfected snails ($p>.05$) (Fig.7). However, mortality of snails was less at 15°C than at 25°C or 30°C ($p<.05$) suggesting that low temperatures, by reducing the metabolic rate of snails, may

Figure 6. Egg production by infected and uninfected Vallonia pulchella at three constant temperatures. Each symbol represents the mean number of eggs/snail-day for four colonies; the arrows indicate the end of the first larval moult. The medians given are the median number of eggs in all colonies for each date prior to the first larval moult.

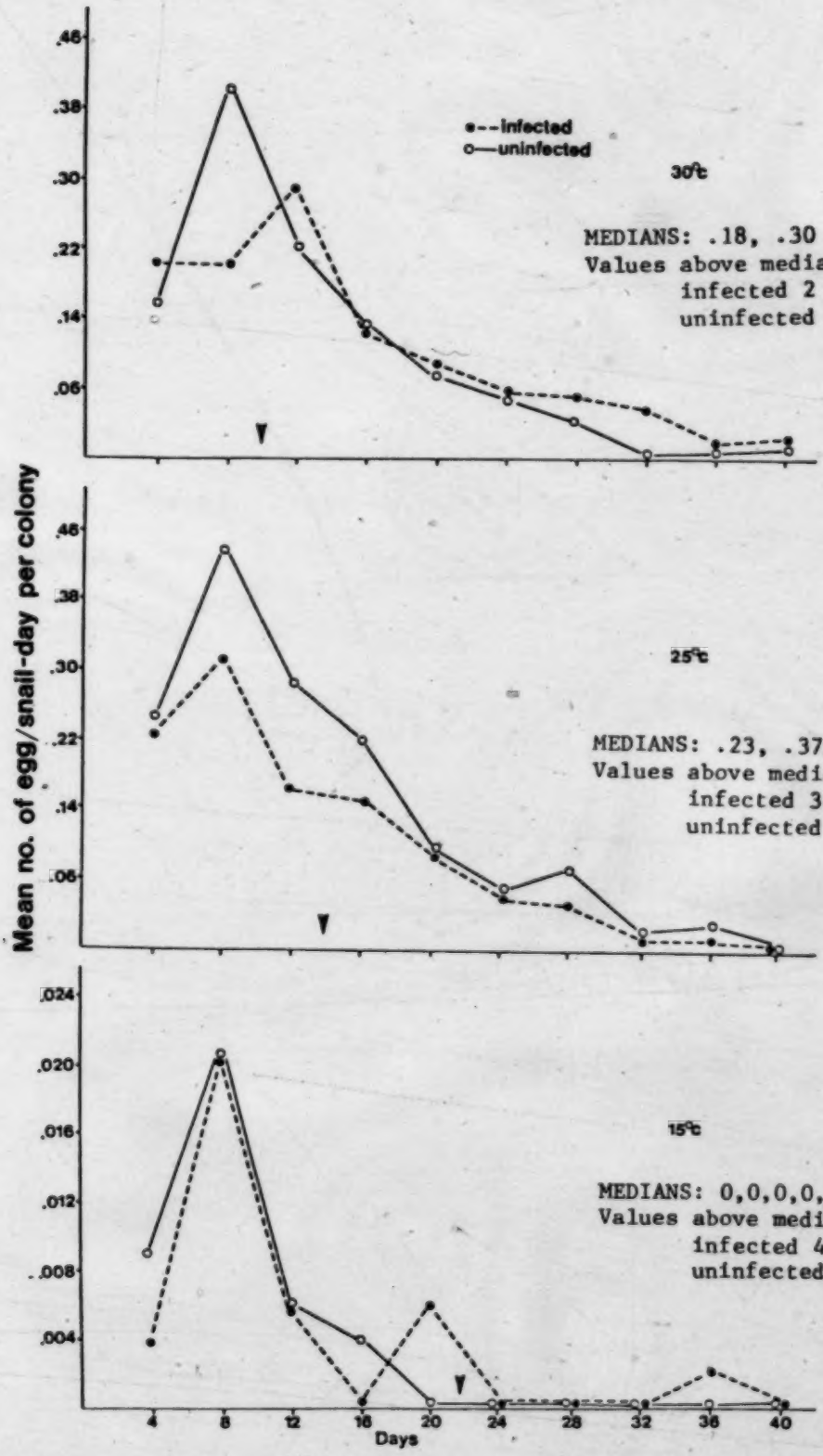
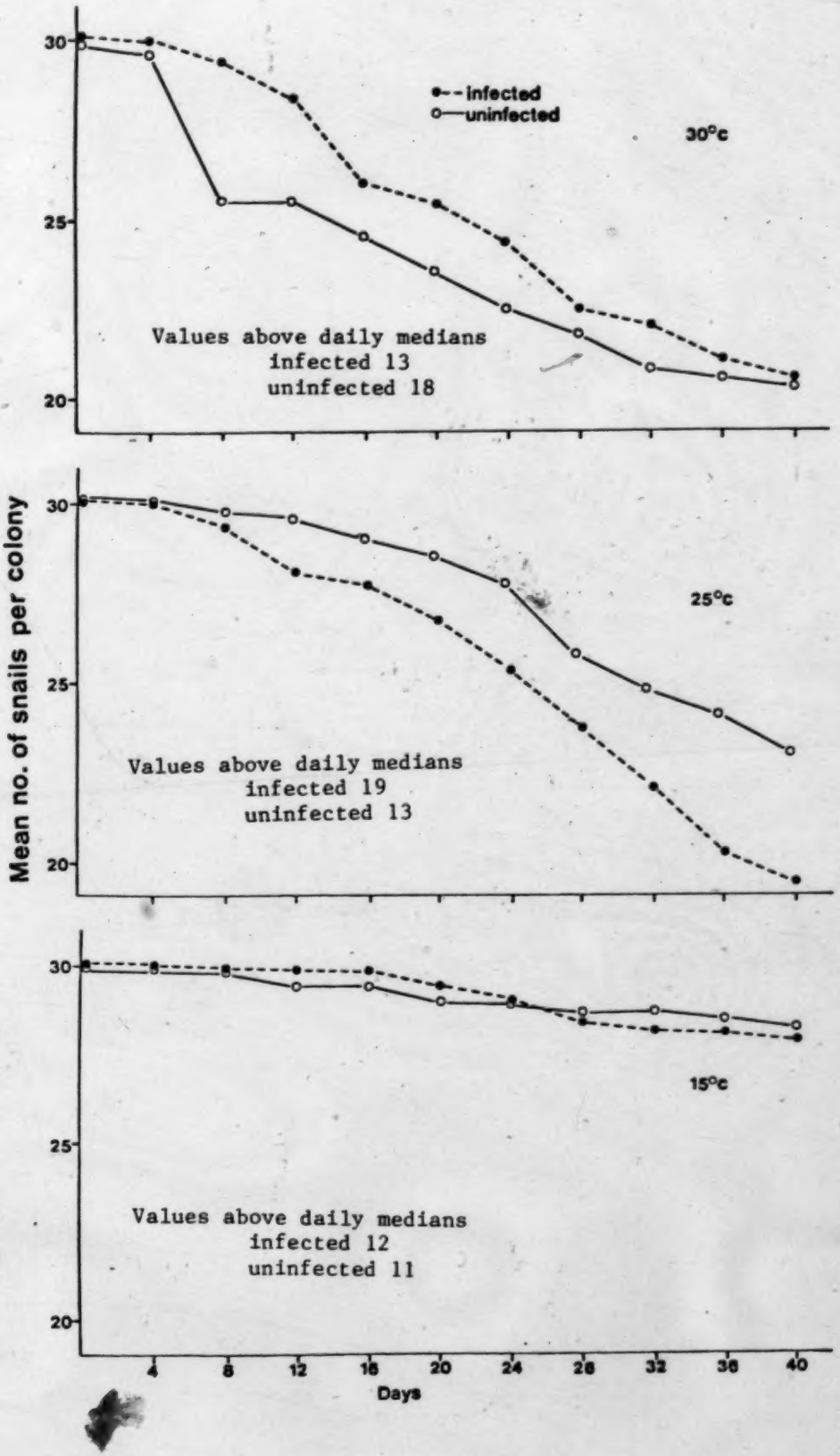

.46
.38
.30
.22
.14
.06
•---infected
o—uninfected
30°c
MEDIANS: .18, .30
Values above medians:
infected 2
uninfected 5
Mean no. of egg/snail-day per colony
.46
.38
.30
.22
.14
.06
25°c
MEDIANS: .23, .37, .22
Values above medians:
infected 3
uninfected 8
.024
.020
.016
.012
.008
.004
15°c
MEDIANS: 0,0,0,0,.025
Values above medians
infected 4
uninfected 6
4
8
12
16
20
24
28
32
36
40
Days

Figure 7. Mortality of infected and uninfected Vallonia pulchella at three constant temperatures. Each symbol represents the mean number of live snails for four colonies. The medians given are the median number of live snails in all colonies.


infected
uninfected
30°c
Values above daily medians
infected 13
uninfected 18
25°c
Values above daily medians
infected 19
uninfected 13
15°c
Values above daily medians
infected 12
uninfected 11
Mean no. of snails per colony
30
25
20
4
8
12
16
20
24
28
32
36
40
Days

have favored longer survivorship.

Protostrongylus infection of snails did not influence the growth rates of their offspring (Fig.8)(see Appendix II). At 25°C, there was no significant difference between the growth rates of snails coming from an infected colony and those coming from an uninfected one ($p>.05$). Eggs from infected and uninfected snails incubated at 10°C did not hatch and those incubated at 15°C hatched but had a low survival rate suggesting that optimal temperatures for hatching and growth were similar to those for maximal egg production.

EFFECT OF *PROTOSTRONGYLUS* ON BEHAVIOR OF *V. PULCHELLA*

Three questions were asked in the experiments on the effects of parasitism on snails when tested on a heat gradient: is movement of infected snails impaired? do infected snails get trapped more or less often than uninfected snails? and is their temperature preference altered? In all three experiments, regardless of whether or not snails were started in the middle of the heat gradient, the warm side, or the cool side, there was no difference between the number of infected and uninfected snails moving along the heat gradient (Table 3). However, significantly more infected snails went to a temperature region and returned ($p<.025$) indicating that those snails wandered more in unfavourable areas and were less trapped than uninfected snails. However, the temperature preference of infected

Figure 8. Growth rates of offspring of infected and uninfected Vallonia pulchella, at 25°C. The symbols represent the mean shell diameter and the lines represent the standard deviation of the means.

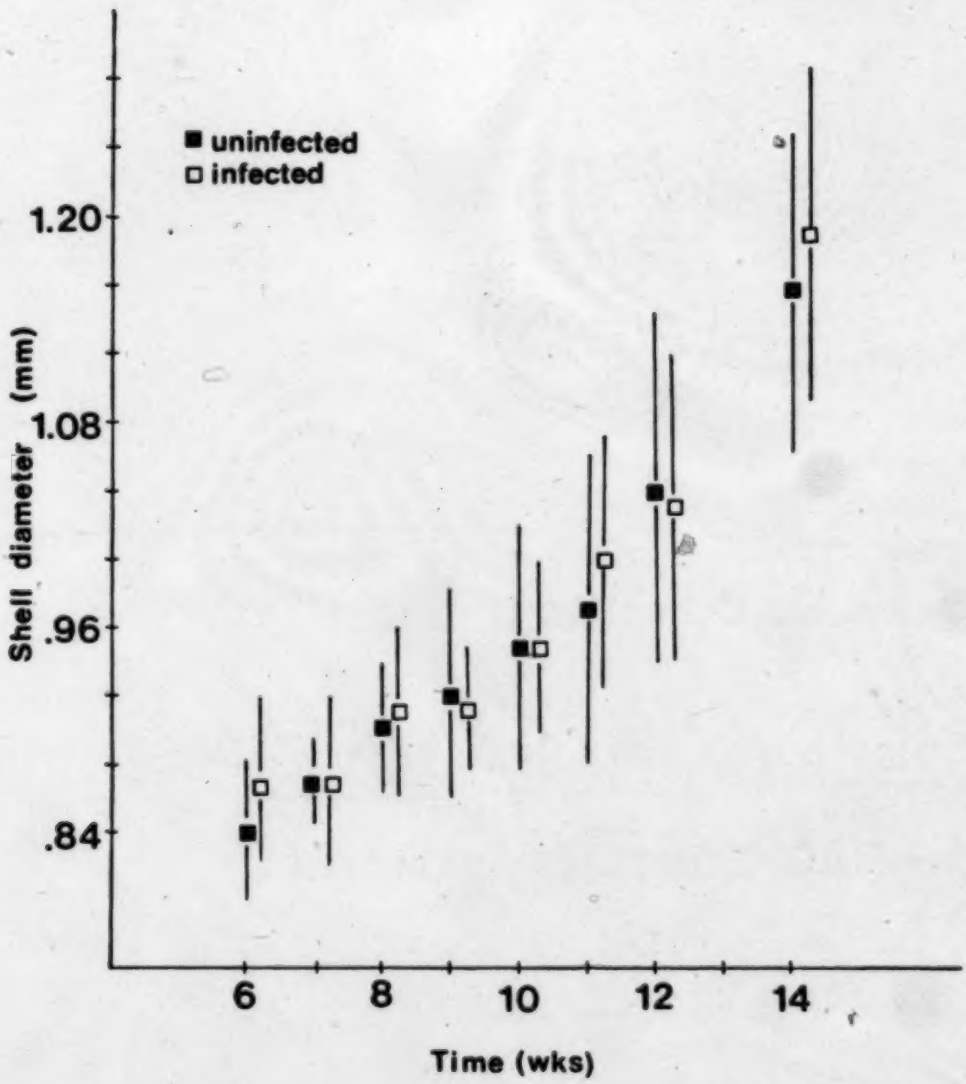


Table 3. Response of infected and uninfected snails to a heat gradient.

Time (min)	No. of snails moving to hot side: uninfected 1[a]	uninfected 2	infected 1	infected 2	No. of snails moving to cold side: uninfected 1	uninfected 2	infected 1	infected 2	Total uninf.	Total infected
Started in the middle of the heat gradient										
15	1	6	2	5	1	5	1	0		
30	3	7	1	6	2	4	2	1		
45	4	7	2	5	2	5	1	0		
60	5	6	2	4	2	7	2	2		
75	6	5	2	7	2	7	1	2		
No. moving	6	7	3	9	2	8	3	3	23	18
No. returning	0	2	1	2	0	1	2	1	3	6
Total no. moving	6	9	4	11	2	9	5	4	26	24
Started in the cold side of the heat gradient										
15	1	0	0	3	0	0	0	1		
30	3	2	1	4	0	0	0	1		
45	9	4	2	7	0	0	0	1		
60	12	5	4	8	0	0	0	2		
75	12	9	5	9	1	1	0	0		
No. moving	12	9	5	9	1	1	0	2	23	16
No. returning	0	0	0	0	0	0	0	2	0	2
Total no. moving	12	9	5	9	1	1	0	4	23	18
Started in the hot side of the heat gradient										
15	5	11	3	6	0	0	2	1		
30	10	12	4	5	1	0	1	4		
45	12	12	3	8	1	0	2	3		
60	13	14	4	6	2	0	2	3		
75	12	12	6	7	3	0	0	4		
No. moving	13	14	7	10	3	0	3	5	30	25
No. returning	1	2	1	3	0	0	3	1	3	8
Total no. moving	14	16	8	13	3	0	6	6	33	33

[a] 1 and 2 are two groups of 15 snails each

snails was not altered by parasitism. When data for infected and uninfected snails were tested separately, neither showed a temperature preference. However, when data for infected and uninfected snails were combined and tested, there is a significant preference for the warm side ($p<.05$). When they were placed at the cool end of the gradient, they migrated to the warm end; snails placed in the warm side tended to stay there. Since there were no signs that movement of snails in the warm end was inhibited, the results indicate that snails (infected or not) preferably moved over a warm substrate than over a cool one.

When responses of infected and uninfected snails to light and dark conditions were tested, infected snails were more active than uninfected snails. Thus, when snails were started in the dark, two moves were observed among uninfected snails from the dark to the light side or vice-versa, versus 18 moves from infected ones, and when snails were started in the light, 26 moves were observed among uninfected snails versus 47 from infected ones (Table 4).

When snails were placed in dark conditions, most snails, infected or uninfected, stayed there, but more infected snails wandered into the lighted zone ($p=.016$). Only one uninfected snail moved into the light zone, but returned to the dark zone by the next observation period whereas 12 infected snails wandered in the light zone and 6 returned to the dark zone. When snails were started in the

Table 4 . Response of infected and uninfected snails to light. Each colony consisted of 10 snails

Snails started in the dark

	Uninfected snails				Infected snails			
	No. of snails in the light				No. of snails in the light			
Time (min)	Colony 1	2	3	4	1	2	3	4
15	0	0	0	0	0	0	1	0
30	0	0	0	0	1	1	1	1
45	0	0	0	0	2	2	1	1
60	0	0	0	0	2	1	2	1
75	0	1	0	0	2	3	2	1
90	0	0	0	0	2	0	1	1
105	0	0	0	0	2	0	1	3
120	0	0	0	0	3	0	1	2
move to light	0	1	0	0	3	4	2	3
move to dark	0	1	0	0	0	4	1	1
total moves	0	2	0	0	3	8	3	4

Snails started in the light

	Uninfected snails				Infected snails			
	No. of snails in the dark				No. of snails in the dark			
15	3	5	5	6	3	1	4	7
30	3	5	5	7	3	3	5	8
45	4	5	6	7	5	2	7	7
60	4	6	7	7	5	2	3	6
75	4	6	7	7	6	3	7	6
90	4	6	7	6	5	2	6	8
105	4	6	7	6	3	2	5	8
120	4	6	6	6	3	3	5	6
move to light	0	0	1	1	3	2	6	4
move to dark	4	6	7	7	6	5	11	10
total moves	4	6	8	8	9	7	17	14

light zone, more infected snails (32) than uninfected snails (24) moved to the dark zone, with only two uninfected snails but 15 infected ones returning back to the light zone. Therefore, when testing light-dark preferences of snails, significantly more infected snails went to a light region and returned indicating that those snails crawled more in unfavourable light areas. Thus, although *Protostrongylus* infected snails preferred dark conditions just like uninfected snails, infected snails tended to move more.

It is difficult to assess whether or not *Protostrongylus* infection altered the rate of movement of *V. pulchella*. Although the total distance covered by the 20 infected snails did not differ significantly from that covered by the 20 uninfected snails ($U=178$, $p>.05$) (Table 5), infected snails stopped and restarted more frequently than uninfected snails. Furthermore, as can be seen in Figure 9, both infected and uninfected snails' activity peaked before the first half of the experiment and decreased during the second half but patterns of activity appeared to differ between parasitized and non-parasitized snails, with parasitized snails moving more, earlier.

The average number of infected snails per colony in the leaf litter after 90 minutes did not differ significantly from that of uninfected snails ($U=24$, $.10>p>.05$) (Table 6). Furthermore, once a snail, infected or not, was in the leaf litter, it stayed there for the duration of the experiment (only one snail, an uninfected one, migrated out of the leaf

Table 5 . Rate of movement of infected and uninfected V. pulchella measured over 12 consecutive five minute periods.

	Infected snails	Uninfected snails
Total distance (mm)/snail		
median	112	93
range	0-608	0-750
Mean distance/period snails moved		
median	3.2	3.2
range	0-11.1	0-13.6
No. stops and restarts	9	3
No. periods snails moved		
median	7	6
range	0-12	0-12

Figure 9. Mean distance covered by infected (n=20) and uninfected snails (n=20) at each five minute period over 60 minutes.

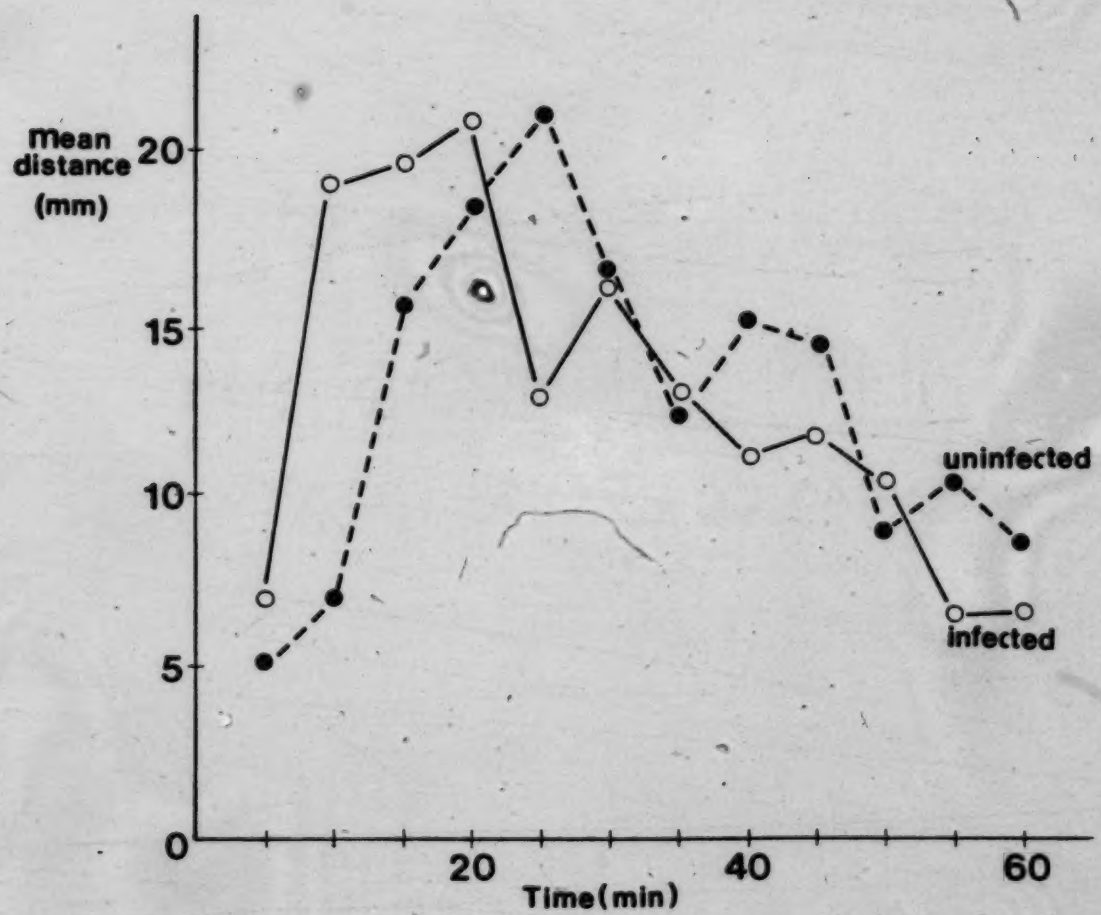


Table 6. Attraction of infected and uninfected snails to leaf litter.

Time (min)	Colony 1 n=10	2	3	4	5	6	7	8	9	10	Total no. of snails moving during period
	Number of snails not in the leaf litter										
	Uninfected snails										
15	10	10	10	9	10	10	9	9	7	10	6
30	9	8	9	7	8	9	10	6	3	10	17
45	8	6	8	3	7	6	10	5	2	8	16
60	8	6	8	3	7	4	9	5	2	8	3
75	8	6	7	3	7	4	9	5	2	8	1
90	8	6	6	3	6	4	9	4	2	8	3
	Infected snails										
15	10	8	10	6	6	8	10	9	9	8	16
30	9	0	6	6	4	5	9	7	9	6	23
45	9	0	5	5	1	5	9	6	7	5	9
60	8	0	2	3	1	4	9	6	6	4	9
75	7	0	2	3	1	3	8	6	2	3	8
90	7	0	2	3	1	2	7	6	2	1	4

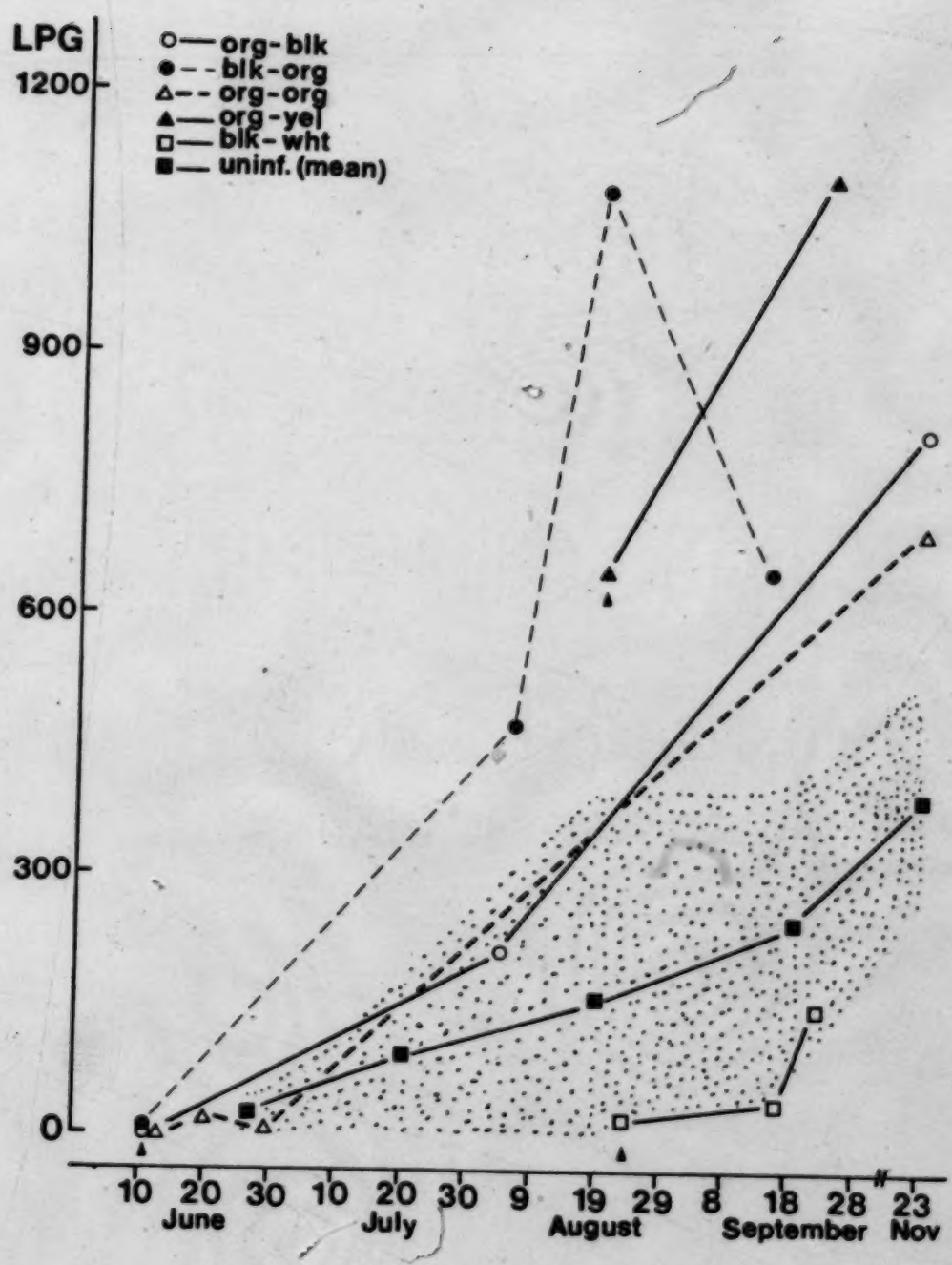
litter). However, the total number of infected snails moving to the leaf litter (69) was significantly greater than the total number of uninfected snails (44) ($p<.01$), and in all but one time interval, the number of infected snails moving to the leaf litter was greater. Therefore, parasitism seemed to have some effect on snails seeking leaf litter.

EXPERIMENTAL INFECTION OF BIGHORN LAMBS

Two experimental lambs, Blk-Wht and Org-Yel, were experimentally infected in August; no fecal samples could be collected from them after the experimental infections would have been patent. Therefore, it is impossible to assess whether or not these infections took. All other experimental lambs shed significantly more larvae in their feces than naturally infected lambs (Fig.10,11) . The highest larval output reached by an experimental lamb in 1982 (dose, 125-150 L_3s) was 1090 LPG (Larvae Per Gram of feces), and in 1983 (dose, 1000 L_3s) the highest output was over 5000 LPG. In addition, as can be seen by comparing figures, the mean August-September larval output for experimental lambs infected with 1000 L_3s was significantly higher than for experimental lambs infected with 125-150 L_3s ($p<.05$) suggesting that a larger intake of L_3s led to a greater establishment of worms in the lungs.

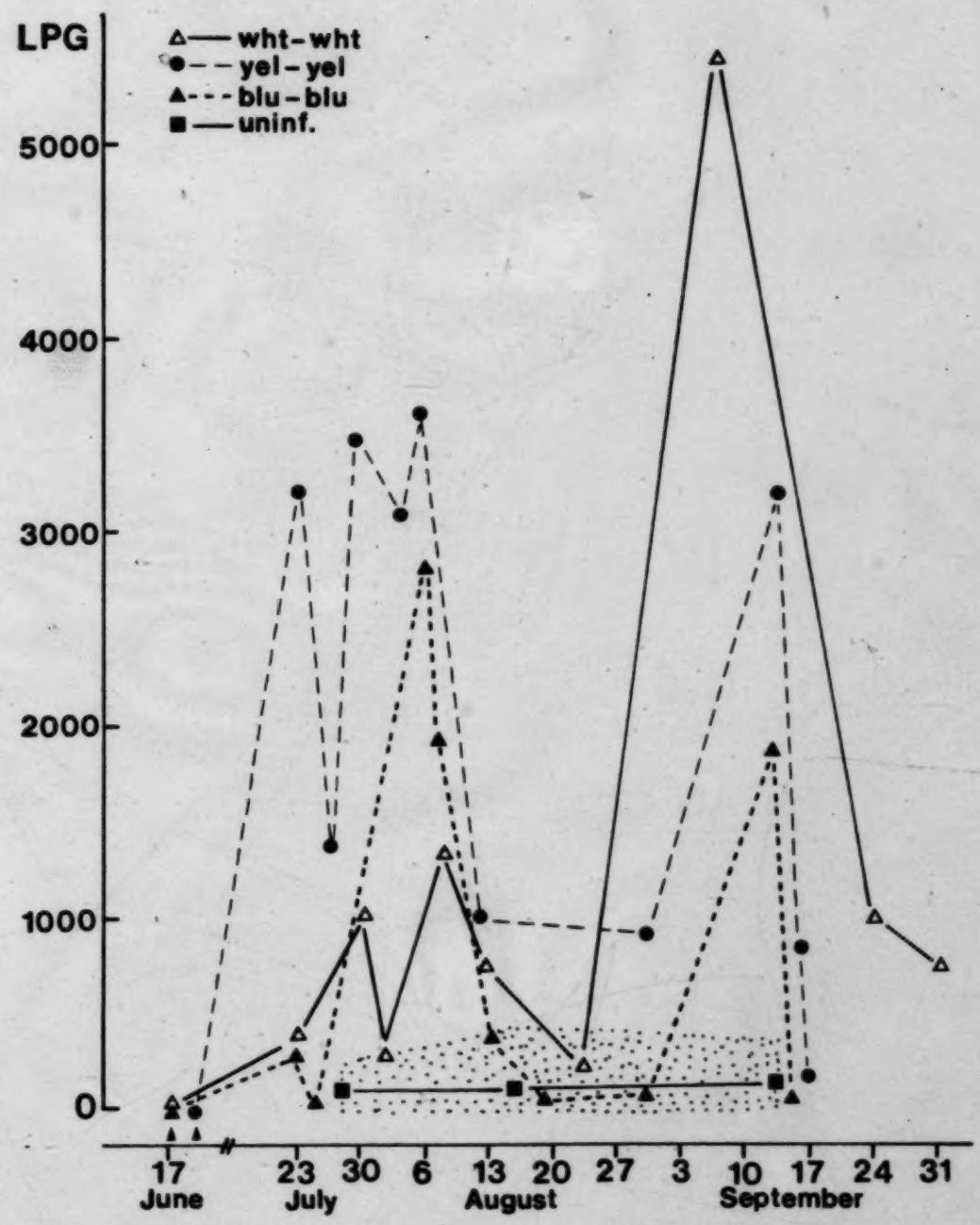
In 1983, larval shedding of all three experimental lambs exhibited a bimodal pattern: the first peak appeared immediately post-patency, and the second peak at the time of

Figure 10. Comparison of larvae per gram of dried feces (LPG) of experimentally infected and control lambs (Ram Mountain, 1982). The solid squares and the dotted area represent respectively the monthly mean larval outputs and the standard deviation of control lambs. The arrows represent the date of infection of experimental lambs.


LPG
1200
900
600
300
0
org-blk
blk-org
org-org
org-yel
blk-wht
uninf. (mean)
10 20 30 10 20 30 9 19 29 8 18 28 23
June
July
August
September
Nov

1982

Figure 11. Comparison of larval outputs (LPG) of experimentally infected and control lambs (Ram Mountain,1983). The solid squares and the dotted area represent respectively the monthly mean larval outputs and the standard deviation of control lambs. The arrows show dates of infection of experimental lambs.


LPG
wht-wht
yel-yel
blu-blu
uninf.
5000
4000
3000
2000
1000
0
17
June
23
30
July
6
13
20
27
August
3
10
17
24
31
September

1983

immediately post-patency, and the second peak at the time of weaning (September). The two peaks were separated by a period of two to three weeks when larval shedding was very low, suggesting that the lambs were able to respond to the infection and were thus immunocompetent to lungworms at the time of infection.

Only two naturally infected lambs in 1982 and one in 1983 had high larval outputs. Org-Yel (Fig.10) was shedding over 600 LPG in late August when it was experimentally given 150 L_3s; one month later, his larval output was 1100 LPG. Blk-Yel, a lamb not exposed experimentally, was passing 874 LPG in his feces in August 1982. This lamb was abandoned at approximately three weeks of age in early June. He remained small during the summer and died the following winter. Blk-Blk, a naturally infected lamb in 1983, had a larval output of almost 1000 LPG on August 29. Two weeks later, his output dropped by half. He appeared healthy.

Even though the numbers of L_1s in the feces of experimental lambs were abnormally high for summer months, the lambs did not exhibit obvious differences from naturally infected lambs in play or resting time and did not show clinical signs of pneumonia such as scruffy hair coat or coughing. In addition, experimental lambs did not differ significantly in weight (Table 7, $U=11$, $p>.10$). Experimental lamb Yel-Yel infected with 1000 L_3s was the only lamb in 1983 to suffer a mild short-term bout of contagious ecthyma around its nose.

Table 7. Weights of experimental and control lambs in August 1983 (Ram Mountain).

Lambs' I D.	Sex	Weight (kg)	Date of measurement
Experimental lambs			
Wht-Wht	M	22.0	August 19
Blu-Blu	F	22.5	August 23
Yel-Yel	M	24.0	August 29
		$\bar{x}$ = 22.8 ± 1.0	
Naturally infected lambs			
Wht/Org	M	23.5	August 18
Unmarked	F	22.0	August 19
Org/Yel	F	20.0	August 19
Yel/Wht	F	18.0	August 19
Blk/Blu	M	26.0	August 23
Blu/Blk	M	26.5	August 23
Blk/Wht	M	29.7	August 27
Org/Wht	F	22.5	August 27
Blk/Blk	M	24.5	August 28
Yel/Org	M	28.0	August 28
Yel/Blk	M	29.5	August 28
		$\bar{x}$ = 24.6 ± 3.8	

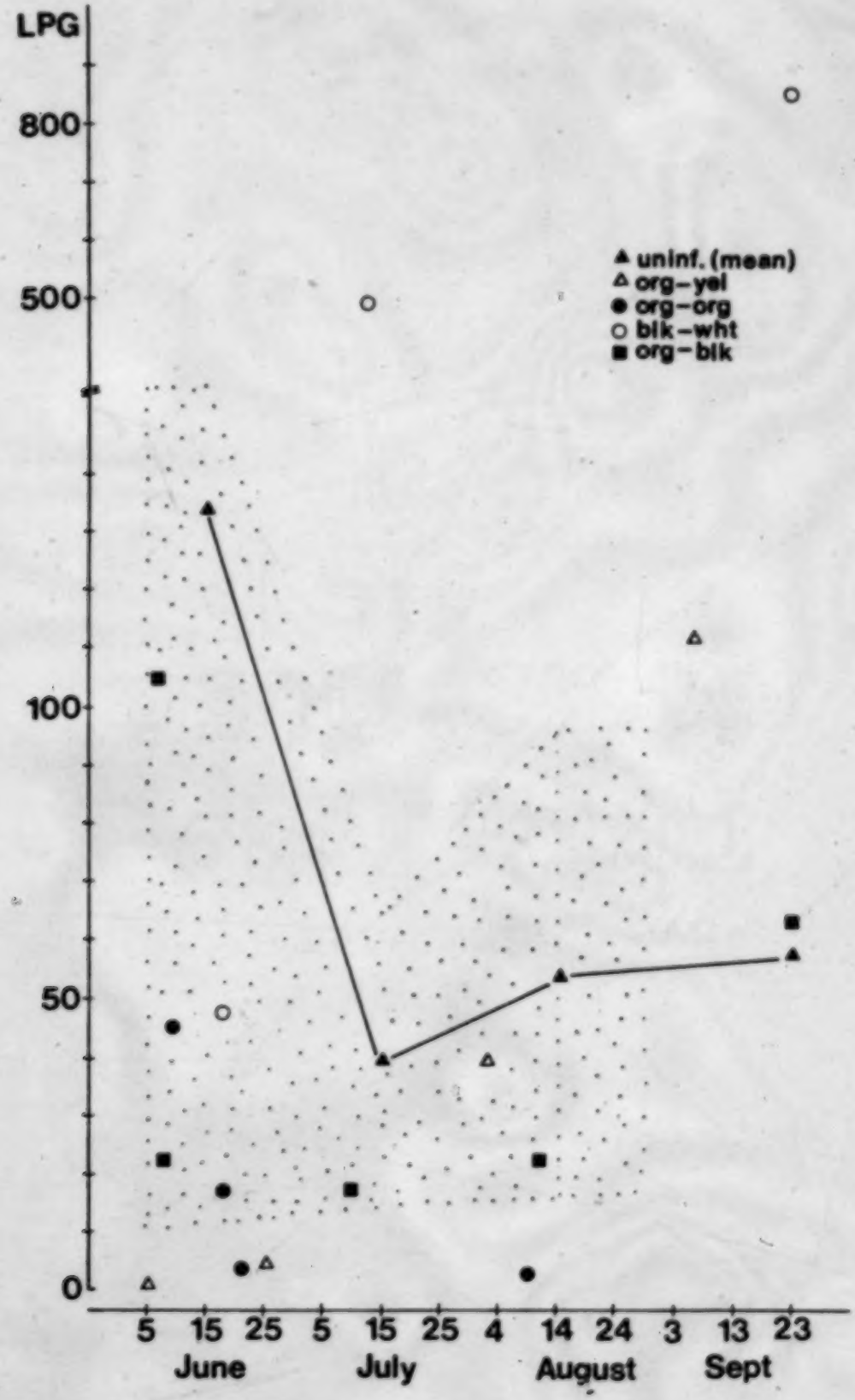
During the fall of 1982 and the winter of 1982-1983, all experimental lambs were relocated and observed monthly. Unfortunately, fecal samples could not be collected during the winter. The physical condition of all experimental lambs appeared excellent and similar to that of naturally infected lambs. The carcass of Blk-Org, which had been partially eaten, was found on July 24, 1983. The cause of the death was unknown, but the lamb had been seen in good health throughout the winter and several times in June up to 40 days prior to discovery of the carcass. The carcass was not suitable for further examination.

During the summer of 1983, fecal samples were collected from the four remaining experimental yearlings (sheep infected experimentally as lambs the previous summer). Larval shedding, a year post-infection, did not appear different from those of naturally infected yearlings (Fig.12) except for Blk-Wht one of two lambs infected in late summer.

All three experimental lambs exposed to 1000 L_3s in 1983 were examined within 36 days of infection; all had increased larval output at that time. Lamb Yel-Yel best exemplified this by apparently responding to exposure of 1000 L_3s on June 18 with a larval shedding over 3000 LPG (Fig.11) on July 23, 1983. This suggests that the prepatent period for *Protostrongylus* spp. is less than 35 days.

In addition, the L_1s of *Protostrongylus* were found in the feces of three of eleven lambs sampled between June 10

Figure 12. Larval outputs (LPG) of experimentaaly infected (all symbols except solid triangles) and control lambs of 1982 a year post-infection (Ram Mountain). The solid triangles show the mean monthly larval outputs of control yearlings; the dotted area represents the standard deviation of those means.


LPG
800
500
100
50
0
▲ uninf. (mean)
△ org–yel
● org–org
○ blk–wht
■ org–blk
5 15 25 5 15 25 4 14 24 3 13 23
June
July
August
Sept

and June 19, 1982; the lambs were approximately three weeks of age. This provides circumstantial evidence for transplacental transmission of lungworms.

IV. DISCUSSION

MODE OF ENTRY

First-stage *Protostrongylus* larvae used two modes of entry to infect *V. pulchella*; most directly penetrated the surface epithelium of the foot, the mantle and probably the proboscis, but some were ingested, then penetrate the mucosal epithelium of the esophagus or intestine. My observations on developing *Protostrongylus* in snails showed that second and third-stage larvae were present only in the foot muscle. Therefore, larvae penetrating tissues other than those of the foot muscle must migrate to the foot muscle to develop or else perish.

Kassai(1958) showed that first-stage protostrongylid larvae (*Cystocaulus ocreatus*) ingested by *Helix* species snails did not survive. Most were either passed out in the feces or digested; those that penetrated the intestinal walls got stuck near the outer surface of the latter, where they degenerated. However, snails of the genus *Helix* are very large compared to *V. pulchella*, and the distance the larvae would have to migrate is much greater. In this study, the evidence was inconclusive. I observed no signs of degeneration; larvae remained apparently viable and in good condition for at least three days in several snail tissues. Such larvae may degenerate later, or may migrate to the foot prior to development. The few L_1s observed at 9-72 hours post-exposure in tissues other than those used for

penetration suggest that the larvae do migrate; the relatively constant number in the foot muscle over the first three days suggest they may not, or that they may take considerable time before migrating. If they do migrate, migration itself is probably effected rapidly because of the small size of the snail.

The fact that the number of larvae per snail increased with time indicated that infection did not stop when snails were removed from the source of infection. Larvae for continued exposure may have come from one (or both) of two sources: larvae present in the gut of some snails could have been excreted and infected other snails, or larvae could have been present, but undetected, in the mantle cavity of the snails. In those snails fixed early, such larvae may have been washed away in the fixative, whereas in those snails fixed late such larvae may have had time to infect them. The former hypothesis seems unlikely, since all larvae in the gut were counted - and therefore could not account for an increase in the number of larvae in snails examined later. However, the latter hypothesis is supported by the increased number of larvae in the mantle wall, as well as the evidence for continued larval penetration of the snail's mantle wall up to 72 hours post-exposure.

Different metastrongyloid nematodes may use either the oral route (passive mode) or penetration (active mode) to infect their gastropod intermediate host (Table 8). The mode of entry used by a given species may be dictated by factors

Table 8. Mode of entry and method of exposure for several nematode-gastropod combinations.

SPECIES	MODE	METHOD	INTERMEDIATE HOST	SOURCES
Aelurostrongylus abstrusis *	A	* 2	Helix aspersa	Hamilton (1969)
" pridhami	A-P	2	Physa integra, Deroceras gracile, Discus cronkhitei, Zonotoides arboreus Succinea ovalis	Anderson (1962)
Anafilaroides rostratus	A		Helix aspersa, Mariella dussumieri Achatina fulica, Laevicaulis alte	Seneviratna (1959)
Angiostrongylus cantonensis	A-P		Agriolimax laevis, Onchidium sp Limax arborum	Mackerras & Sandars (1955)
" "	A-P		Achatina fulica	Cheng & Alicata (1965)
" "	A-P	2	Biomphalaria glabrata	Yousif & Lammler (1977)
" "	A-P	2	" "	Harris & Cheng (1975)
" "	P		" "	Richards & Merritt (1967)
" dujardini	P	1	Planorbis planorbis, P. corneus Lymnaea corvus, L. stagnalis, L. peregra Planorbarius corneus, Hygromia sp. Retinella incerta, Biomphalaria glabrata	Drozdz et al. (1971)
Cystocaulus ocreatus	A	3	Helix pomatia, H. obina	Kassai (1958)
Filaroides martis	A		Physa integra, Deroceras gracile	Anderson (1962)
Morerastrongylus andersoni	P	1	Lymnaea stagnalis, Planorbarius corneus	Petter & Cassone (1975)
Muellerius capillaris	P		---	Hobmaier & Hobmaier (1934)
" tenuispiculatus	A		Cepaea vindobonensis, Succinea putris	Svarc & Zmoray (1974)

SPECIES		MODE	METHOD	INTERMEDIATE HOST	SOURCES
Parelaphostrongylus	odocoilei	A	3	Deroceras laeve, Vitrina limpida	Platt & Samuel (1984)
"	"	A-P	3	Zonotoides arboreus	" "
"	tenuis	A-P	2	Discus cronkhitei, Zonotoides arboreus Deroceras gracile, Stenotrema fraternum, Triodopsis albolabris, Anguispira alternata	Anderson (1963)
Protostrongylus	boughtoni	P	3	Vallonia pulchella	Kralka (1983)
"	rufescens	A		---	Hobmaier & Hobmaier (1934)
Protostrongylus and/or P. rushi	stilesi	A-P	3	Vallonia pulchella	This study

*A = active mode (penetrate snail)
P = passive mode (ingested by snail)

1 = intermediate hosts in contact with larvae-infected food
2 = intermediate hosts in contact with an aqueous solution containing larvae
3 = intermediate hosts are allowed to crawl on a filter paper wetted with a larvae-infected aqueous solution

such as the nematode species itself, the intermediate host species, the nematode-host species combination, or the method of exposure of the intermediate host to the larvae.

The mode of entry of at least ten species of metastrongyloids has been studied in more than one intermediate host species. Six nematode species use a consistent mode of entry in several intermediate hosts (e.g., *Muellerius tenuispiculatus* invade actively *Cepaea vindobonensis* and *Succinea putris*) whereas four species use either mode to invade their intermediate hosts. It is difficult to assess why such variation in mode of entry exists. However, from the present studies, there does not seem to be a factor in the nematode that stipulates the mode of entry.

Several gastropod hosts have been used to investigate the mode of entry of more than one nematode species. There are at least two species of intermediate hosts that are consistently invaded by the same mode (or consistently by both modes) of entry by different parasites (e.g., *Zonotoides arboreus* is invaded by both modes by *Aelurostrongylus pridhami*, *Parelaphostrongylus odocoilei*, and *Parelaphostrongylus tenuis*; *Helix aspersa* is actively invaded by *Aelurostrongylus abstrusis* and *Anafilaroides rostratus*). However, there are five species of intermediate hosts invaded by different modes of entry as reported by several studies (e.g., *Achatina fulica* is invaded by *Anafilaroides rostratus* actively and by *Angiostrongylus*

cantonensis by both modes). Such variation may be due to some properties of the snail. Joyeux and Gaud (1946) (but see Kassai,1957, for a contrary opinion) stated that *Muellerius* larvae are able to "penetrate only small species [snails] covered with loose mesoderm", a characteristic that is common in small and in young snails. Thus, Hobmaier and Hobmaier (1934) who obtained only a passive mode of entry by *M. capillaris* may have used adult snails that had a tough mesoderm, whereas Svarc and Zmoray (1974) may have used juvenile snails with a loose mesoderm to obtain an active mode of entry by *M. tenuispiculatus*. Therefore, there does not seem to be a consistent mode of entry into a specific intermediate host.

The reports in which both modes of entry have been used are not consistent with the hypothesis that the mode of entry may be specific to a particular host-parasite combination. In addition, the only combination that has been studied by several workers is the *Angiostrongylus cantonensis-Biomphalaria glabrata* combination, in which no consistent mode of entry was found.

Three principal methods have been used to expose snails to first-stage nematode larvae. First, the intermediate hosts have been put in contact with larvae-infected food (feces or lettuce) (method 1 in Table 8). In the two studies using this method, ingestion is reported as being the only mode of entry. Second, the intermediate hosts are placed in an aqueous solution containing larvae (method 2 in Table 8).

Anderson (1962), Harris and Cheng (1975), and Yousif and Lammler (1977) noted that both modes were used, whereas Hamilton (1969) indicated penetration of the foot as the only mode. Yousif and Lammler (1977) infected both active and anesthetized snails in an aqueous solution containing *A. cantonensis* L_1s. They observed a significantly greater number of larvae in active snails than in anesthetized snails, which had non-functioning buccal masses, and concluded that the oral route was the primary mode of entry. In the third method, the intermediate hosts are allowed to crawl on a filter paper wetted with a larvae-infected aqueous solution (method 3 in Table 8). Using this method, Kassai (1958) observed active penetration, Kralka (1983) observed ingestion as the only mode of entry whereas Platt and Samuel (1984) observed both modes for *Zonotoides arboreus* and only penetration of the foot for *Deroceras laeve* and *Vitrina limpida*, and I have observed both modes of entry. Therefore, unless first-stage larvae are mixed with food, in which case larval ingestion seems to be the mode of entry, the method of exposure does not seem to provide a clear pattern in the pathway used by larvae to invade their intermediate host.

Although the mode(s) of entry of several parasitic nematode-intermediate host systems have been elucidated, the mechanisms governing mode(s) remain unclear. The use of an active mode has major advantages: larval stages may respond to some environmental stimuli in ways that usually bring

them closer to a potential intermediate host (MacInnis,1976). Therefore, any ability of first-stage *Protostrongylus* larvae to respond to stimuli in the environment would promote infection of snails and represent a significant adaptation toward effective transmission to their definitive host bighorn sheep. Kassai (1958) has found such ability: snail's mucus trails have been shown to induce a response by protostrongylid larvae to favor penetration. However, in this case, because of the small size of the larvae and thus their unlikeliness to travel any distances, contact with the intermediate host's mucus is probably a random event. Other potential advantages of the active mode include an ability of the larvae to identify the host as an appropriate one thereby avoiding loss through failing to develop in an unsuitable host, and an ability to gain entry in a site suitable for development in the host. However, a main disadvantage of the active mode may be a shorter free-living life span resulting from the more rapid use of energy reserves.

Probably the main advantage of the passive mode is a longer free-living life span resulting from a conservation of energy. However, because the parasite may not respond to any environmental stimuli, and encounter of the parasite with the host being a chance event, the possibilities that the parasite contacts a wrong host are greater. As well, if the parasite is ingested and needs to develop in tissues other than those of the gastro-intestinal tract,

considerable energy may be required by the parasite to attain its site of development.

Since *Protostrongylus* larvae are able to use either mode of entry to invade their intermediate hosts, they might have evolved adaptations that would combine advantages of the active and passive modes. Such adaptations may include: a relatively long free-living life span, use of the passive mode during periods of larval inactivity due to unfavorable environmental conditions, and invasion of small snails to promote larval development in a suitable site (foot) when either mode is used.

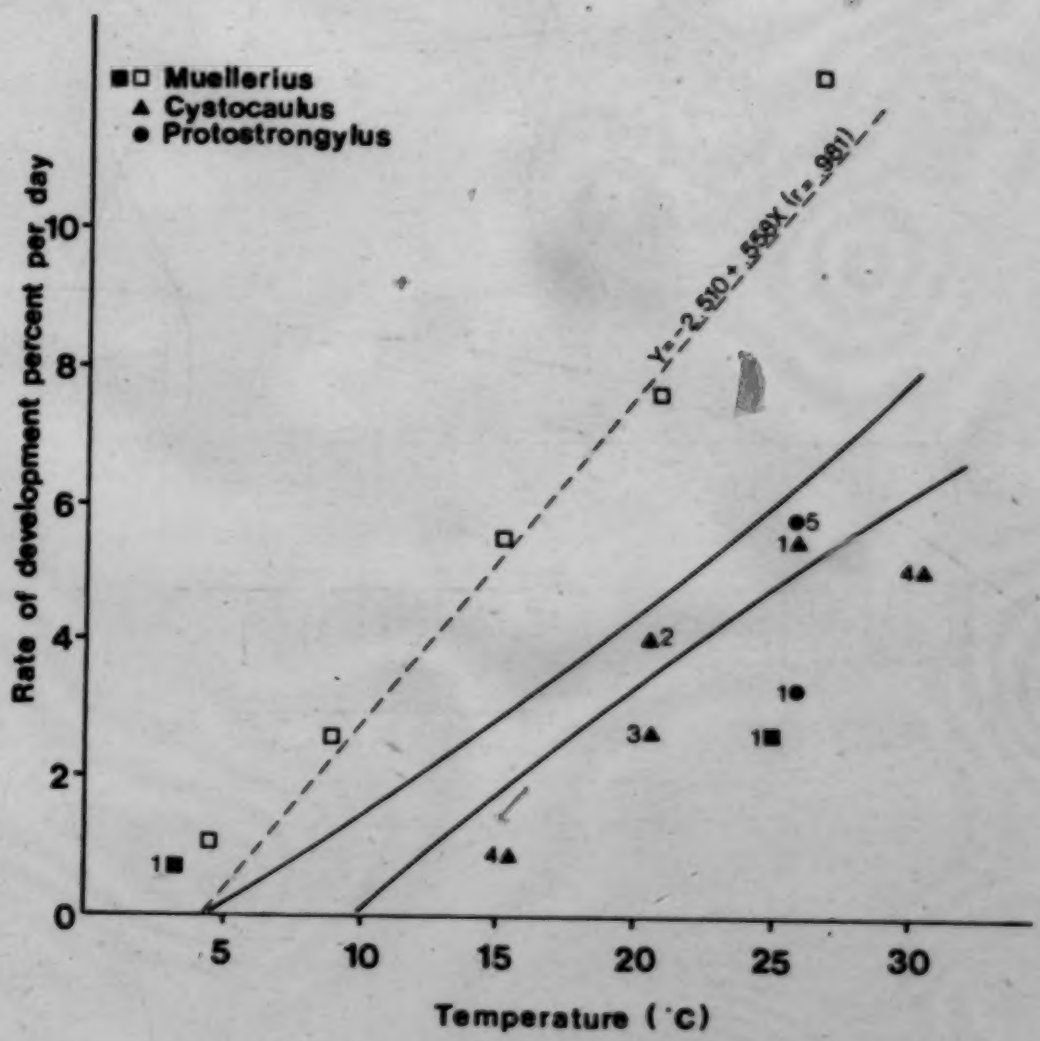
EFFECT OF TEMPERATURE ON DEVELOPMENT OF *PROTOSTRONGYLUS* IN *V. PULCHELLA*

Once *Protostrongylus* larvae have invaded their intermediate hosts, temperature probably becomes the most important environmental factor affecting their development. *Protostrongylus* larvae developed rapidly at high temperatures in *Vallonia pulchella* but development was markedly slowed down at lower temperatures until a thermal threshold (8°C) was reached where no larval development proceeded. The experiments in which infected snails were kept at cool temperatures (10°C) for a length of time and then transferred to an optimum temperature indicated that the retardation of development at lower temperatures did not involve a diminution in viability or in the developmental capacity of the larvae. In all cases, development of larvae

proceeded immediately and at a faster rate than expected. The cause of this accelerated development is not clear. A similarly accelerated development has been observed for *Polymorphus marilis* in *Gammarus lacustris* by Tokeson and Holmes (1982). Perhaps some larval physiological functions are conducted more efficiently than others at low temperatures or more efficiently during or after a change of temperatures than if kept at constant temperatures.

Several other studies on lungworm-gastropod systems have reported the number of days required by various lungworms to reach the third larval stage in intermediate hosts. When the rates of development converted from the developmental time (in days) reported in those studies were plotted against temperature, most of the points fell outside the 95% confidence intervals for the regression of the rate of development of *Protostrongylus* larvae in *Vallonia pulchella* (Fig.13). Generally, the rate of development of *Protostrongylus* larvae is the same or higher than those reported for other lungworms except for *Muellerius capillaris* (Rose, 1957). However, the fact that *Protostrongylus* larvae have a thermal threshold of 8°C is shared by other lungworm-gastropod systems. Gerichter (1948,1951) and Kassai(1958) observed that *Cystocaulus ocreatus* do not undergo moulting at temperatures of 4-8°C. *Protostrongylus rufescens* (Gerichter, 1951), *Protostrongylus kochi* and *Aelurostrongylus abstrusus* (Gerichter, 1948) also do not develop at temperatures below 8°C. However,

Figure 13. Rate of development to third larval stage of various lungworms compared to that of Protostrongylus stilesi and/or P. rushi. The dotted line is the regression for Muellerius capillaris as calculated from data (open squares) in Rose (1973). The solid lines enclose the 95% confidence limits of the regression of Protostrongylus spp (see fig 5) (Sokal and Rohlf, 1969). Solid symbols show rates calculated from data in the literature. 1. Gerichter (1951), 2. Cabaret and Dakkak (1979), 3. Kassai (1957), 4. Davtyan (1945), 5. Joyeux and Gaud (1946).


■□ Muellerius
▲ Cystocaulus
● Protostrongylus
Y= -2.510 + .559X (r= .98**)
Rate of development percent per day
Temperature (°C)
0
2
4
6
8
10
5
10
15
20
25
30
1■
4▲
3▲
1■
1●
▲2
1▲
●5
4▲

Muellerius capillaris can develop at 4-8°C (Gerichter, 1951), and Rose's data provide a calculated threshold of 4°C. However, caution must be exercised when rates near the thermal threshold are extrapolated from the regression line since Stinner *et al.* (1974) have demonstrated that rates obtained from the regression line near the threshold value are less than the actual ones. This is due to the fact that rates of development measured at all temperatures provide a sigmoid curve instead of a linear one.

The apparent discrepancies in the rate of development between species of lungworm larvae may be attributable to one or more of three procedural factors or three real factors. The first procedural factor is the lack of precision in temperature measurements (or times of development) as recorded by several authors, in particular Gerichter (1951). His rates of development have been calculated from results of experiments run at temperatures varying between 20 and 30°C, calculated at the mean of 25°C. Any difference between the actual mean temperature and that used in the calculations could lead to marked differences in rates of development. On the basis of Rose's study, a span of 10°C in recording temperatures could account for almost a doubling in the rate of development of larvae. A second procedural factor that may be involved is the difference between the time taken to reach the third larval stage and that taken to reach the infective stage. Rose (1957) showed that the number of days required by *Muellerius capillaris*

larvae to reach the third larval stage and the infective stage as described by Gerichter (1948) are different, especially at lower temperatures. Thus, it is possible that different authors used different end points, resulting in discrepancies in rates of development. If this is the case, it may explain the differences between Gerichter's work (1951) and Joyeux and Gaud's work (1946) on *Protostrongylus rufescens* (Fig.13). Thirdly, some of the studies reported minimum times to development to the third (or infective) stage, not T_{50}. Most of the other studies did not mention the criteria used in deciding the end point, therefore small variations between studies based on this probably exist. My own observations show that at high temperatures, minimum time to development to the third stage is similar to T_{50}. However, at low temperatures (15°C), minimum time to development can be faster than T_{50} by at least three days (about 7% of T_{50}). Therefore, this factor can account for only small variations between studies.

One of the real factors contributing to discrepancies in the rate of development of lungworm larvae may be the "appropriateness" of the intermediate host. As described by Gerichter (1948), an "appropriate" snail host is one in which infection invariably succeeds and the nematode's larval development is rapid, whereas "inappropriate" snails are those in which only a small percentage of the snails can be infected, and the development of the invading larvae proceeds slowly. Gerichter did not provide data to support

this difference in developmental rate. However, different authors have reported different rates of development for the same parasite in different intermediate hosts, as shown in figure 13. In particular, Gerichter's work is inconsistent with that of other authors; the "appropriateness" of the gastropod host may have influenced the developmental time of the larvae.

A second real factor that may produce differences in rate of development is associated with the physiological state of the snail and of the larvae. Svarc and Zmoray (1974) reported that *Muellerius tenuispiculatus* larvae that concentrated in the surroundings of the sole glands (bottom of the foot) of *Cepaea vindobonensis* developed normally to the infective stage whereas those located in the upper foot (which lacks sole glands) were delayed in development. They attributed this phenomenon to the important role played by sole glands in the nutrition of *M. tenuispiculatus* larvae. Davtyan (1940 in Kassai 1958) claimed that the nutritional condition of snails do not, in general, influence the rate of development of protostrongylid larvae. However, Kassai (1958) concluded that in some instances larvae are unable to develop, or develop slower, in snails having markedly reduced metabolism, which often occurs when snails are in a torpid state (e.g., aestivation) since the nutritive level of the tissue fluids may be so reduced that larval development cannot be sustained.

Lastly, geographical isolates of some species of lungworms may have developed into different strains adapted to different conditions and with different rates of development in their different gastropod hosts. This could account for some of the variation observed between Gerichter's work and that of others since he (and Joyeux and Gaud) worked in a very hot and arid region, Palestine, whereas most of the others worked in cooler regions of Europe or North America. However, this feature cannot explain the difference between his work and that of Joyeux and Gaud.

Factors such as crowding and multiple infections do not seem to influence rate of development. Although crowding has been found to retard development of some larval parasites, namely *Diphyllobothrium dendriticum* (Guttowa, 1967), Halvorsen (1976), in a review paper, concluded that this is not the case with nematodes. Concurrent infections of different species of parasites in a host have also been shown to have deleterious effects for the development of some pseudophyllidian cestodes (Guttowa, 1967) but Gerichter (1948) observed that multiple infections of lungworms in snails did not influence their rates of development.

Given all the factors that may influence the development of lungworm larvae in gastropod hosts, it is possible that the details of the rate of development of *Protostrongylus* in other intermediate hosts will not resemble those observed in *Vallonia*. However, the general

pattern presented here seems to be universal, and indicates that under natural conditions, *Protostrongylus* larvae in a snail host probably develop very slowly during cool weather (<15°C) but rapidly at warm ambient temperatures (>25°C), and suggests that no larval development would proceed at temperatures lower than 8°C, precluding development in hibernating snails.

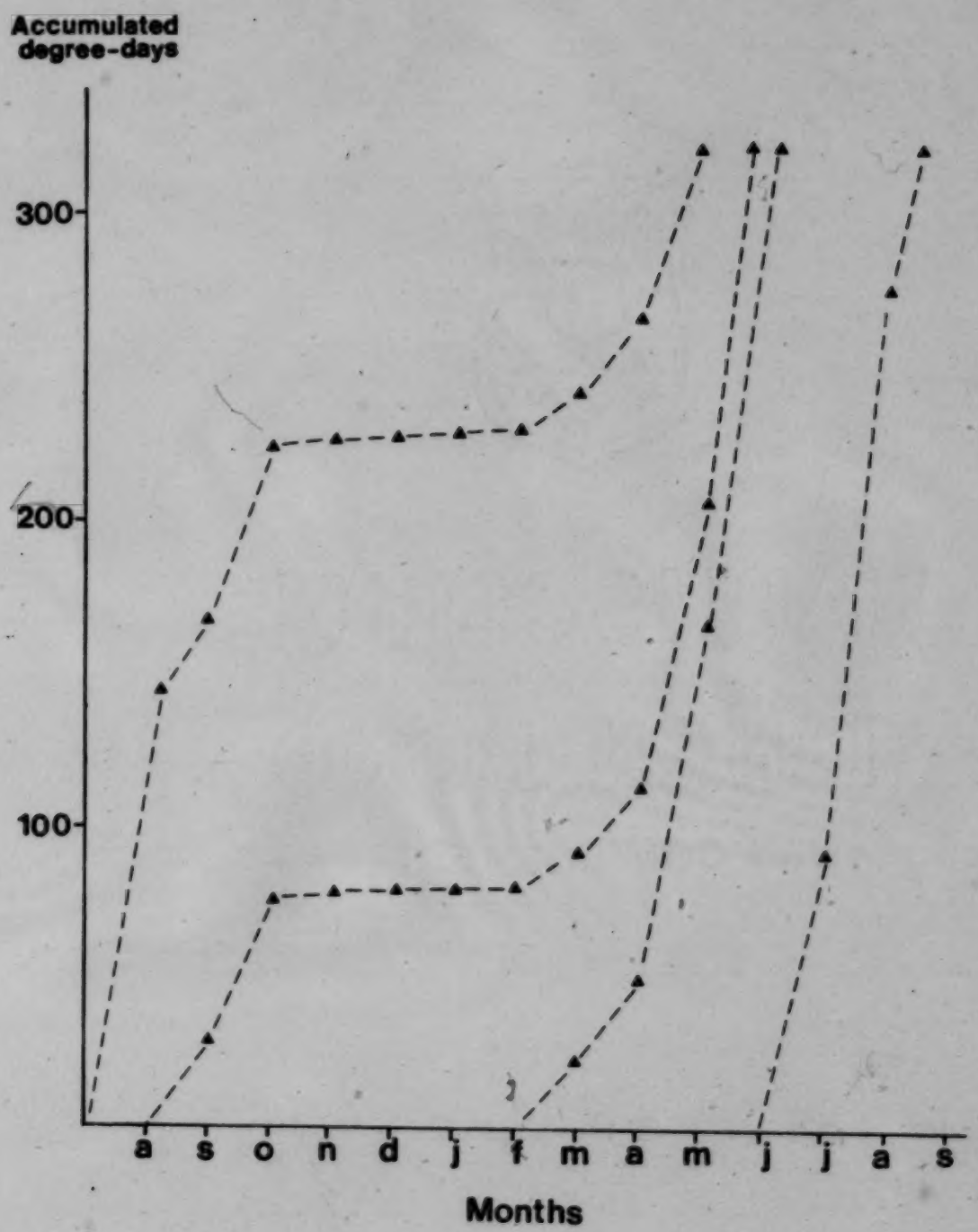
Developmental rates of *Protostrongylus* larvae at different temperatures in the laboratory can be used to calculate expected developmental rates under field conditions if daily soil temperatures are avalaible. Such data were available from an aspen grove on the Sheep River winter range during 1980-1981. Boag and Wishart (1982), in a study of this range, demonstrated that the maximum abundance of potential intermediate host snails was found within forested areas, including the aspen grove where soil temperatures were taken. This winter range is a prime habitat for bighorn sheep from September to May. During this period, the sheep often congregate in the aspen groves.

Calculations of developmental rates applied to field conditions are as follows: field temperatures at 4-hour intervals were obtained from thermograph records. The temperature excess over the thermal threshold (8°C) provided the number of degree-periods accumulated by worms in snails over each four-hour period. Degree-periods were divided by six to give degree-days and totalled until the thermal constant (305 degree-days) was reached.

These methods indicate that almost no development occurs during the cool weather of spring or fall, but that very rapid development takes place in the summer. The data also indicate (Fig.14) that, to be infective to sheep by late May, when the sheep are leaving the wintering grounds, larvae must have infected the snails by early September of the previous year, and survived overwinter. To be infective by late September, when snails are becoming inactive, larvae must have infected the snails by mid-July. These time periods may be overestimates since, as demonstrated, worms kept in snails kept at low temperatures develop faster than usual when transferred to higher temperatures.

The snail intermediate hosts on the Sheep River winter range are viable for at least a year (Boag and Wishart,1982); once snails acquire larvae, they retain most of those larvae for life (personal observation). These two features suggest that infected snails should be available to bighorn sheep on their winter range in both spring and fall. However, since populations of snails suffer overwinter mortality, then start expanding during the summer to reach a peak in late summer-early fall (Boag and Wishart,1982), the number of snails on the winter range is probably considerably greater in the fall than in the spring. Also, because of rapid larval development at high summer temperatures, a greater proportion of these snails are likely to carry infective larvae in the fall. Consequently, infection of sheep is most likely to occur in the fall.

Figure 14. Predicted development of *Protostrongylus* larvae (L_1s to L_3s) in *Vallonia pulchella* throughout the year based on soil temperatures measured in an aspen grove at Sheep River, Alberta, to calculate the mean accumulated degree-days (solid symbols) at the end of each month.


Accumulated degree-days
300
200
100
a s o n d j f m a m j j a s
Months

Significant infection of sheep on the summer range is unlikely since the habitat on this range is generally unsuitable for snail populations, sheep occur at lower densities, and numbers of larvae shed by the sheep are generally low (Uhazy *et al.*, 1973).

EFFECT OF *PROTOSTRONGYLUS* INFECTION ON FECUNDITY, MORTALITY AND GROWTH RATES OF *V. PULCHELLA*

Snail fecundity was directly related to temperature, as temperature increased so did egg-laying. Whitney (1938) indicated that egg-laying in *V. pulchella* may take place whenever the animal is in a good state of nutrition and external conditions, such as temperature and humidity, favorable. Eggs laid under dry conditions are more likely to perish since they are susceptible to desiccation; for eggs laid during cold weather, the retarding effect on development and hatching would prolong their exposure to other hazards. It is thus to the advantage of snails to lay eggs in warm and humid conditions to favor survival of the offspring.

Egg-laying decreased with time probably because, at best, egg production is limited by the large size of the eggs (approximately 0.5mm in diam.) in proportion to the size of snails (less than 2.0mm), which necessitates their being laid singly. Therefore, snails may become reproductively exhausted from too intensive egg-laying.

However, at all temperatures, egg-laying peaked at day 8 and then decreased remarkably. Some hypotheses can be put forward. First, the transfer of snails from a terrarium with abundance of leaf litter and lettuce, to a finger bowl with a limited amount of leaf litter may have caused a sudden increase in egg-laying as the snails initially put in the finger bowls had plenty of food but as the resources became depleted, reproduction slowed. This is very likely, because egg-laying by snails kept in the terrarium always increased following addition of leaf litter and lettuce. Second, the accumulation of by-products (e.g., feces, mucus) in bowls after some time may have had an inhibitive effect on reproduction. As well, stress induced through handling snails, as described below, may have prevented reproduction.

Since the snails kept in bowls were maintained at relatively constant humidity level, similar to that of the terrarium, it is unlikely they suffered from desiccation after day 8 or 12. It is also improbable that a change in temperature, when snails were transferred from the terrarium to bowls, may have triggered and then reduced egg-laying. Snails transferred from the terrarium (which was kept at room temperature, approximately 22°C) to bowls kept at 25°C would have shown a different egg-laying pattern than those at 15°C and 30°C.

It was shown that *Protostrongylus* larvae did not influence fecundity of their intermediate host *V. pulchella*. Thus far, most information on parasitic effects on molluscan

intermediate hosts life cycle parameters comes from trematode infections which have been shown to exert a negative influence on their intermediate host's reproductive system (Wright,1971). Lungworms can be expected to cause fewer problems to host reproduction than trematodes for two reasons. First, lungworms develop to the infective stage in the foot of their gastropod host and are not in direct contact with the host's reproductive organs, thus reducing their chance of damaging the reproductive organs. And second, as mentioned earlier, the greatest energetic demand on the snail host probably occurs when lungworm larvae undergo development up to the first ecdysis whereas in trematodes, invading larvae continue to reproduce, thus making higher energetic demands on the host, for a long period of time. Therefore, the nematode-intermediate host system probably does not need to derive advantages that a trematode castrator would such as increased host survivorship and growth to augment energy availability for the parasite to improve its own fitness (Baudoin,1975).

Snail mortality was independent of time. Two major stress factors can be responsible for that other than mortality from natural causes. First, the colony was searched thoroughly every four days for egg collection, thus disturbing snails by misplacing them while searching. Second, every four days all snails were picked up with forceps and placed on a wet slide to verify viability. In some instances, this procedure inflicted shell damages fatal

to the snails. These two stress factors may also explain the reduction in snail fecundity.

There was no difference in the rate of mortality between infected snails and uninfected snails. Several studies, in addition to this one, have reported that lungworm infection does not influence mortality rate in gastropods. Gerichter (1948) observed that snails infected with as many as 200 larvae were apparently not harmed, and their mortality rate was as low as that of uninfected snails. Kassai (1958) experimentally infected *Helix* spp. with over 1000 protostrongylin larvae without observing any ill effects on the snails. Cabaret and Dakkak (1979) showed that *Cochlicella* snails heavily infected with *Cystocaulus* and *Neostrongylus* also had a low mortality rate. Thus, it is recognized that lungworm larvae do not cause fatal damage to their intermediate host at least in the laboratory.

Growth rates after six weeks of age of offspring of infected snails were not different from those of offspring of uninfected snails, indicating that infected snails are capable of laying eggs from which healthy animals can grow. It is unlikely that some differences in growth rates immediately after hatching or within six weeks of birth may have occurred and gone undetected since measurements taken six weeks post-hatching showed no differences. Nevertheless, data suggest that the quality of eggs of infected snails is not impaired by parasitism. Whitney (1938) observed that juvenile *Vallonia* snails grow under favorable conditions at

about 0.2mm per week (diameter of the shell). This growth rate is more rapid than the one observed in this study (approximately 0.03mm/wk). Since growth of young is dependent on activity (thus on temperature) and food (Pillmore,1955), different experimental conditions are likely to yield different growth rates. However, the growth rate observed in this study is much slower than the one observed by Whitney (1938), suggesting that the experimental conditions in this study were not favorable. Thus, quality of eggs coming from infected parents must have been relatively good, since the offspring were able to develop as well as offspring coming from uninfected parents even in 'unfavorable' conditions.

EFFECT OF *PROTOSTRONGYLUS* ON BEHAVIOR OF *V. PULCHELLA*

So far, it has been shown that *Protostrongylus* larvae do not have a significant effect on the life cycle parameters of the intermediate host *Vallonia pulchella*. However, the results of the experiments designed in the behavior study disclosed some behavioral modification of *V. pulchella* by *Protostrongylus* larvae. The responses of infected snails to three stimuli (heat gradient, light conditions, and presence of vegetation) were generally consistent: infected snails were less often trapped than uninfected snails in the heat gradient experiment and in the light condition experiment, and their response to the stimulus (heat and light) was the same since they both

preferred to crawl over a warm substrate and wander in dark areas. Also, both infected and uninfected snails sought the cover of vegetation. However, rates of movements of infected and uninfected snails for all four experiments seem to differ with infected snails being generally more active than uninfected snails. Thus, *Protostrongylus* infection did not have an effect on responses of snails to stimuli but seemed to have decreased their chances of being trapped in an area.

When infected snails were allowed to crawl freely for a period of time, the distance they covered was the same as the one covered by uninfected snails. However, infected snails stopped, rested, and started crawling again more often than uninfected snails. Perhaps, presence of larvae in the foot of snails is irritating or is draining energy from the snails so much that it obliges them to stop and rest more often.

Holmes and Bethel (1972) outlined four strategies used by parasites to increase predation on the intermediate hosts. These are reduced stamina, increased conspicuousness, disorientation, and altered responses. However, such strategies are used primarily when the intermediate host is actively preyed upon by the definitive host. Since accidental ingestion of snails by bighorn sheep is considered the mode of infection of the definitive host, only two of the outlined strategies could be applicable to this system: disorientation and altered responses.

Disorientation of infected intermediate hosts is associated with parasites damaging the central nervous system or major sensory receptors of the host, so that a disoriented host would wander into unusual habitats, or make itself more available or conspicuous to a predator. Altered responses of an infected host are changed responses to some environmental stimuli. A good example of such effects is *Dicrocoelium dendriticum* in ants (Anokhin, 1966 in Holmes and Bethel, 1972). Infected ants respond to dropping temperatures by clinging to grass tips during the cold hours of the day instead of returning to the colony as do uninfected ants. Such response enables the infected ants to become more available to accidental ingestion by herbivores and thus enhance parasite transmission to the definitive host. In the *Protostrongylus-Vallonia* system, either a disorientation or altered responses of infected snails such as tolerance of colder temperatures, brighter light conditions, more exposed areas might have been anticipated because of the high percentage of infected sheep. However, parasitism had no effect on the light and temperature preferences of snails, but did decrease their rate of trapping so that the infected snails were more frequently found in unfavorable areas.

It is difficult to assess whether these minor behavior modifications induced by lungworms in their intermediate hosts would actually enhance transmission.

EXPERIMENTAL INFECTION OF BIGHORN LAMBS

Results of the experimental infections of lambs with *Protostrongylus* spp. were assessed by the numbers of larvae recovered from their feces using the Baermann's technique. However, because there is considerable variation in larval outputs from one animal over time one must be careful when assessing such results. For example, Rose (1959) shows that, although the number of superficial nodules of *Muellerius capillaris* in the lungs of domestic sheep provides a good approximation of the number of worms in the lungs, the number of L_1s in feces does not correlate with the number of adults in the lungs. According to Rose, this can be partly explained by the fact that a high proportion of nodules are made up of single worms, thus no copulation can take place. This of course results in no L_1s in the feces. Forrester and Senger (1964) also reported considerable variation in the number of *Protostrongylus* larvae within an individual dropping of a bighorn, and between samples of droppings collected daily from the same animal. Therefore, results obtained from the experimental and naturally infected lambs should be analyzed with some caution especially where there are relatively few samples from each lamb. Despite this caution, it is obvious that exposure of bighorn lambs to 1000 L_3s of *Protostrongylus* spp. in 1983 resulted in establishment of adult parasites in the lungs, as indicated by the very large numbers of L_1s shed in their feces. The smaller numbers of L_1s shed in the feces of lambs given

125-150 L_3s in 1982 are less convincing, but still suggest that worms did establish and reproduce successfully in the lungs, at least of the three lambs exposed early in the summer.

The similarity in larval outputs (LPG) of experimental sheep (low dose group) one year post-exposure to those of naturally infected sheep is probably related to the well-known seasonally cyclical larval outputs of *Protostrongylus* spp. in bighorn sheep (Uhazy *et al.*, 1973; Jorgenson and Wishart, 1982) with a typical low shedding of larvae during the summer and fall, and a high shedding in late winter and early spring. Two explanations for the reduced shedding in summer and fall are likely: first, that adult worms are short-lived and most die by summer. Second, that established adult worms are either reproductively inactive, i.e., they do not lay eggs during the summer, or eggs and L_1s are trapped in the tissues and do not leave the lungs during the summer. However, whether or not the worms are short-lived, some intrinsic regulation of the activity of the worms in the lungs must be taking place, since the period of high larval shedding corresponds to the time of the year when bighorns are in their worst body condition, but not to the time expected according to the life cycle. Because ingestion of infected snails probably takes place in early fall, as shown above, and the prepatent period is less than 35 days, a high larval shedding would be expected to follow in late fall or early winter, at a time when bighorn

sheep are still in good body condition.

Larval shedding of all three experimental lambs in 1983 exhibited bimodal curves, suggesting that bighorn lambs are immunologically competent to fight lungworm infection by the time they are one month old. An initial infection of 1000 L_3s probably led to the establishment of adult worms in the lungs and to production of the larvae of the first peak followed by a period of low larval output where the worms might have become temporarily reproductively exhausted and/or were prevented to reproduce by the host immunological response. The second peak corresponds to the time when lambs are weaned: this probably produced an immunological stress that allowed reproduction of the worms or hatching of eggs or release of L_1s.

Such response to infection by young bighorn lambs indicate that they are not immunologically tolerant to lungworms and do not have a reduced ability to respond to the worms. However, much remains to be known about the immunological status of bighorns toward the parasite and it is only through an understanding of the host's defense mechanisms to *Protostrongylus* infections that these and other perplexing features of the lungworm-pneumonia complex can ever be solved.

Generally, prepatent periods of species of *Protostrongylus* are relatively short (see review in Kralka, 1983). For example, in domestic sheep and goats, *Protostrongylus rallietí*, *Protostrongylus rufescens* and

Protostrongylus skrjabini have prepatent periods between 30 and 37 days, which corresponds closely to that observed in bighorn sheep by Spraker (1979) and in this study. However, longer and more inconsistent prepatent periods have been determined in bighorns and bighorn hybrids. For example Pillmore (1956, 1959) and Lange (1973) reported prepatent periods of 60 days and 42-56 days respectively for *Protostrongylus stilesi* and/or *P. rushi* in a bighorn/domestic hybrid and in a bighorn. Prepatent periods were inconsistent (63, 119 and 122 days) in three bighorn/mouflon hybrids infected experimentally with *P. stilesi* (Monson and Post, 1972). It is difficult to explain the variation in the different prepatent periods of *Protostrongylus* spp. observed by some researchers, but perhaps factors such as the definitive host species (hybrids may be inappropriate hosts, hence longer prepatent periods) and its immune status, condition of the ingested larvae, and poor scientific methods are responsible.

Circumstantial evidence for transplacental transmission has been extensively documented (Forrester and Senger, 1963, 1964; Howe, 1965; Uhazy *et al.*, 1973; Gates and Samuel, 1977; Hibler *et al.*, 1972, 1974). However, in several of these reports, lambs thought to have been transplacentally infected with lungworms were older than six weeks. Given a four to five week prepatent period, infections at that age could have occurred through accidental ingestion of infected snails while feeding on

vegetation since lambs can nibble grass within two weeks after birth (Murie, 1944 and Welles and Welles, 1961, in Lawson and Johnson, 1983) and in this study, a lamb abandoned at about three weeks of age was able to survive indicating that such grazing can be extensive.

Spraker (1979) investigated transplacental transmission by necropsying 32 bighorns that included fetuses at 3.5 months of gestation and lambs up to four months of age. He observed a clear pattern of clinical signs, and of gross and histological lung lesions that correlated with the activity and maturation of *P. stilesi* in the lungs of these lambs. Spraker concluded from his necropsies that L_3s of *Protostrongylus* cross the placenta of the adult ewe and enter the fetal liver in the latter stages of pregnancy. These L_3s remain in the liver until parturition after which they migrate to the lungs of the newborn, develop to maturity by 3.5 to 4 weeks and produce thousands of ova by approximately 5 weeks. Assuming that the development of ingested L_3s is the same as transplacentally transmitted L_3s (or possibly slower, if L_3s develop somewhat during migration) shedding of larvae in feces of lambs approximately three weeks old indicates that the lambs acquired lungworm infection prior to birth. However, since it takes approximately 4.5 to 5 weeks for L_1s to appear, the presence of small numbers of larvae in the feces of lambs about three weeks old suggests that the L_1s shed in the feces may have been tranplacentally acquired L_1s and not

those produced by adult worms that developed from transplacentally transmitted L_3s. This possibility is supported by the demonstrated presence of L_1s in the livers and the lungs of fetuses (Gates and Samuel,1977; Festa-Bianchet and Samson, 1984).

The extensive lamb mortality occurring in some American states (see review in Spraker, 1979) has been attributed to *Protostrongylus* lungworms present in sufficient numbers to cause severe lung damage (Spraker, 1979). Hibler *et al.* (1982) speculated that "lambs born in sheep population where verminous pneumonia is responsible for severe lamb mortality frequently are infected with 100 to 500 larvae [L_3s]. The exact number necessary to predispose lambs to fatal verminous pneumonia is unknown, but 100 probably is sufficient". The discrepancy between this statement and my results leads to four hypotheses: 1) different populations of bighorns (or a population under different conditions over time) may be variable in their resistance to lungworm infection, 2) more than one 'strain' of *P. stilesi* and/or *P. rushi* may exist , 3) another agent, bacterial or viral, must be present in addition to the lungworm to induce fatal damage to the bighorn lung, or 4) only a small part of the L_3s given to free-ranging lambs establishes in the lungs.

Stress could be a major component of the first hypothesis. Populations under stress can be more susceptible to disease. Lange (pers. comm.,1979 in Lance,1980) observed that a group of approximately 30 desert bighorns (*Ovis*

canadensis mexicana) in New Mexico suffered an epizootic of contagious ecthyma immediately after capture and confinement. According to Lance (1980) "although documented by case history and observational data only, it is believed by researchers that acute *Pasteurella* sp. pneumonia, seen in confined sheep and the all age die-offs in large free ranging herds, is induced through long-term low level stress". Thus, he speculates that long-term low level stress reduces disease resistance through adrenal cortical stimulation or other mechanisms.

Another component of the first hypothesis is the inherent ability of sheep within certain herds to resist infection better than others. Range conditions and environmental factors mediated by geographic isolation have contributed to the establishment of lo and high quality populations as characterized by Geist (1971). Sheep of high quality populations, in better body condition, may be better able to resist infection with *Protostrongylus* (or other diseases) than sheep of low quality populations.

Geographic isolation may also have favored the evolution of certain genetic pools in bighorn sheep populations that would render them more or less resistant to disease. The existence of clearly defined differences in susceptibility of domestic sheep to nematodes has been shown (Whitlock,1958; Scrivner,1964,1967; Ross,1970; Altaif and Dargie,1976) and, often, the genetically determined resistance to a nematode infection is a function of the

host's immunological responsiveness to the presence of parasite antigens (Hudson, 1973).

It is known that certain breeds of domestic sheep and certain individuals within breeds better survive infections of trichostrongyle nematodes (Wakelin, 1978). It has also been demonstrated that breeds of sheep of hemoglobin type A were more resistant to *Haemonchus contortus* than breeds of sheep of other types (B or HbA/B) (Evans and Whitlock, 1964). Thus, geographic isolation that exists between bighorn herds may have contributed to restrict their genetic pools (Skiba and Schmidt, 1982) so that some populations, just like breeds of domestic sheep, may have evolved with certain physiological traits that would enhance or decrease their ability to fight parasitic infection such as lungworms.

Because of the geographic isolation (e.g., bighorns on the southernmost part of the Brazeau range are totally isolated from other herds in the province), *Protostrongylus stilesi* (or *P. rushi*) may have become a polytypic species, that is, in certain bighorn sheep populations a more virulent strain of lungworms may be present than in the other sheep populations. This phenomenon is currently observed among the different geographic forms of *H. contortus* and the result is bionomic and immunologic differences between geographic strains (Das and Whitlock, 1960; LeJambre and Whitlock, 1968; Crofton and Whitlock, 1969). As for lungworms of bighorn sheep, morphological differences led Honess (1942) to believe that

a third species of *Protostrongylus*, *Protostrongylus frosti*, existed in Wyoming. Although this species is very similar to *P. stilesi*, and is not generally recognized as a distinct entity, the differences between them may reflect strain differences, with greater differences in pathogenicity.

The presence of a bacterium or a virus may be imperative to induce fatal damage to the bighorn lung. Lungworm larvae might only create lesions that will facilitate microbial invasion (Post and Winter, 1957), with a greater number of lesions, associated with more damage by microbes. In Alberta, *Pasteurella hemolytica* Type T (non-hemolytic) have been isolated from lung, throat and nasal swabs (Onderka, pers. comm., 1983) of bighorns in the southern part of the province (Crowsnest Pass herd, Waterton Lakes herd, and Sheep River herd), and in southern British Columbia. Lungworm levels are variable ranging from low in British Columbia to moderate and severe in Alberta (Samuel and Onderka, pers. comm., 1983). All of the sheep infected with *Pasteurella hemolytica* Type t (non-hemolytic) have had serious respiratory problems. This bacterium has not been found in the respiratory tract of any of the 62 bighorns examined from Ram Mountain, although it has been found in the only bighorn from which the tonsils were examined (Onderka pers. comm., 1984)

Since it is not known what proportion of infective larvae ingested by bighorns will become reproductive adult worms in the lungs, it is possible that even a dose of 1000

L_3s may be too small to lead to the establishment of a significant number of worms, to induce pneumonia. This could have been tested by giving a known dose of infective larvae to a lungworm-free bighorn and examining its lungs for adult worms at the time of the prepatent period. No lungworm-free bighorns were available, and the small number of experimentally infected free-ranging sheep precluded killing any to determine the number of adult worms present.

In order to assess which of the hypotheses (or a combination, or all) is (are) correct, more knowledge on the immunocompetency of bighorn sheep is necessary. It is only through an understanding of the immunological mechanisms used by Rocky Mountain bighorn sheep to fight lungworm infection, an infection present in almost every Rocky Mountain bighorn herd in North America, that the lungworm-pneumonia complex can be understood, and perhaps controlled.

V. SUMMARY

There is no strong evidence that *Protostrongylus* lungworms alter the population dynamics of their intermediate host populations or the behaviour of their intermediate hosts, except for rate of activity. Whether or not this enhances transmission to bighorn sheep is questionable. Despite that, the mechanisms of transmission of larvae to the definitive host must be efficient since nearly all Rocky Mountain bighorn sheep in North America are infected with lungworms.

Temperature is probably the most important environmental factor affecting the development of *Protostrongylus* larvae in their intermediate hosts, since at high temperatures the infective stage is attained rapidly whereas at low temperatures almost no larval development occurs. Since snails can be viable for several years and retain lungworm infection once acquired, it is clear that snails containing infective larvae can be available to sheep all year round. However, because of the timing of infection of snails, the rate of larval development in snails (under field conditions), and the density of sheep and of snails, infection of bighorn sheep probably occurs in the fall on the winter range.

Ingestion of high numbers of snails containing infective lungworm larvae does not necessarily lead to fatal verminous pneumonia in bighorn lambs as demonstrated by the results of the experimental infections performed on bighorn

lambs at Ram Mountain. Therefore, the verminous pneumonia that has been responsible for lamb mortality in Colorado is not necessarily a mortality factor applicable to all bighorn herds. There are other factors (e.g., stress, environmental conditions, body condition of the ewe during pregnancy and lactation and perhaps the extent of transplacental transmission) that seem equally important in determining the probability of death.

There is indication that bighorn lambs approximately one month old are already immunologically competent to fight lungworm infection since larval shedding of all three experimental lambs given 1000 L_3s exhibited bimodal curves. This study suggests that the immunological aspects of the lungworm-pneumonia complex are probably the main key features determining the significance of the worms' activity in the lungs. This area of research could yield exciting results and provide insight on the perplexing lungworm problem in bighorn sheep, and perhaps on ways to control it.

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VII. APPENDICES

APPENDIX I. Fecundity and mortality data for infected and uninfected snails at three different temperatures.

15°C

days	UNINFECTED no. eggs	no. snail alive	s-d	eggs/s-d	INFECTED no. eggs	no. snail alive	s-d	eggs/s-d
4	2	30	120	.017	0	30	120	0
	0	30	120	0	0	30	120	0
	0	30	120	0	2	30	120	.017
	2	30	120	.017	0	30	120	0
8	0	30	120	0	2	30	120	.017
	0	30	120	0	1	30	120	.008
	4	30	120	.033	7	30	120	.058
	6	30	120	.050	0	30	118	0
12	0	30	120	0	3	30	120	.025
	0	30	118	0	0	30	120	0
	0	30	120	0	0	30	120	0
	3	30	118	.025	0	29	116	0
16	0	30	120	0	0	30	120	0
	0	29	116	0	0	30	120	0
	0	30	120	0	0	30	120	0
	2	29	116	.017	0	29	116	0
20	0	30	116	0	0	30	120	0
	0	29	116	0	0	30	120	0
	0	30	120	0	3	30	120	.025
	0	29	116	0	0	29	114	0
24	0	28	116	0	0	30	120	0
	0	29	116	0	0	30	118	0
	0	30	120	0	0	30	118	0
	0	29	116	0	0	28	112	0
28	0	28	112	0	0	30	116	0
	0	29	114	0	0	29	116	0
	0	30	120	0	0	29	116	0
	0	29	116	0	0	28	112	0
32	0	28	112	0	0	28	112	0
	0	28	112	0	0	29	116	0
	0	30	120	0	0	29	114	0
	0	29	116	0	0	28	112	0
36	0	28	112	0	0	28	112	0
	0	28	110	0	0	29	116	0
	0	30	120	0	1	28	112	.009
	0	29	116	0	0	28	112	0
40	0	28	110	0	0	28	112	0
	0	27	110	0	0	29	114	0
	0	30	120	0	0	28	112	0
	0	29	116	0	0	28	112	0

25°C

days	UNINFECTED no. eggs	no. snails alive	s-d	eggs/s-d	no. eggs	no. snails alive	s-d	eggs/s-d
4	27	30	120	.225	38	30	120	.317
	32	30	120	.267	21	30	120	.175
	33	30	120	.275	28	30	120	.233
	28	30	120	.233	19	30	120	.158
8	65	30	120	.542	45	30	120	.375
	56	30	118	.475	37	30	118	.314
	49	30	120	.408	40	30	118	.339
	45	30	120	.375	28	30	120	.233
12	55	30	120	.458	32	30	118	.271
	28	29	116	.241	19	29	110	.173
	30	30	120	.250	13	29	114	.114
	24	30	118	.203	13	30	118	.110
16	22	30	118	.186	35	29	116	.302
	47	29	114	.412	14	26	102	.137
	19	30	120	.158	5	28	112	.045
	15	29	114	.132	11	29	116	.095
20	9	29	116	.078	18	29	114	.158
	12	28	112	.107	12	25	100	.120
	16	30	118	.135	5	28	108	.046
	5	28	112	.045	5	29	114	.044
24	14	29	116	.121	7	28	106	.066
	3	28	110	.027	3	25	90	.033
	4	29	114	.035	2	26	104	.019
	9	28	110	.082	6	28	108	.055
28	9	29	114	.079	3	25	98	.031
	6	27	104	.058	1	20	80	.013
	9	28	110	.082	2	26	102	.020
	4	27	98	.041	5	26	104	.048
32	2	28	110	.018	1	24	90	.011
	2	25	94	.021	1	20	70	.014
	1	27	108	.009	2	25	94	.021
	1	22	88	.011	1	26	102	.009
36	2	27	106	.019	0	21	84	0
	0	22	88	0	0	15	60	0
	2	27	106	.019	1	22	88	.011
	3	22	92	.033	1	25	96	.010
40	0	26	104	0	1	21	82	.012
	1	22	86	.012	0	15	60	0
	2	26	100	.020	0	22	86	0
	0	21	84	0	0	23	90	0

30°C

	UNINFECTED				INFECTED			
days	no. eggs	no. snails alive	s-d	eggs/s-d	no. eggs	no. snails alive	s-d	eggs/s-d
4	28	30	120	.230	36	30	120	.300
	15	30	120	.125	24	30	120	.200
	22	30	120	.183	18	30	120	.150
	14	30	118	.119	22	30	120	.183
8	46	30	118	.390	12	30	120	.100
	32	30	102	.314	33	30	120	.275
	57	30	118	.483	12	30	118	.102
	39	29	104	.375	35	30	118	.297
12	29	29	116	.250	25	30	118	.212
	9	21	84	.107	48	30	118	.407
	40	29	116	.345	25	29	116	.216
	17	23	92	.185	36	29	112	.321
16	9	29	114	.079	8	29	112	.071
	11	21	78	.141	18	29	106	.170
	25	29	116	.216	9	29	110	.082
	11	23	92	.120	21	27	108	.194
20	4	28	112	.036	6	27	106	.057
	11	18	68	.162	13	24	96	.135
	7	29	114	.061	7	26	102	.069
	5	23	90	.056	9	27	108	.083
24	16	28	112	.143	3	26	102	.029
	2	16	58	.034	5	24	90	.056
	4	28	112	.036	4	25	100	.040
	1	22	86	.012	12	27	106	.113
28	10	28	110	.091	0	25	96	0
	0	13	50	0	9	21	84	.107
	0	28	112	0	6	25	94	.064
	0	21	82	0	5	26	100	.050
32	0	27	102	0	1	23	90	.011
	0	12	48	0	4	21	82	.049
	0	28	110	0	3	22	88	.034
	0	20	80	0	2	24	96	.021
36	0	24	96	0	0	22	88	0
	0	12	48	0	0	20	76	0
	1	27	108	.009	0	22	86	0
	0	20	78	0	1	24	94	.011
40	1	24	94	.011	0	22	88	0
	0	12	48	0	2	18	76	.026
	0	27	108	0	0	21	82	0
	0	19	76	0	2	23	88	.023

APPENDIX II. Growth rates of offspring born to infected and uninfected *Vallonia pulchella*, at two constant temperatures.

25°C Age (weeks)	offspring born to uninfected *Vallonia* $\bar{X}$ diam (mm) + S.D		offspring born to infected *Vallonia* $\bar{X}$ diam (mm) + S.D	
6	0.84	0.04 n=50	0.87	0.05 n=50
7	0.87	0.03	0.87	0.05
8	0.90	0.04	0.91	0.05
9	0.92	0.06	0.91	0.04
10	0.95	0.07	0.95	0.05
11	0.97	0.09	1.00	0.07
12	1.04	0.10	1.03	0.09
14	1.16	0.09 n=32	1.19	0.10 n=27
15°C				
6	0.56	0.05 n=11	0.52	0.02 n=6
7	0.61	0.05	0.57	0.04
8	0.64	0.04 n=5	0.62	0.04 n=5

APPENDIX III. Larval counts (LPG) of experimental lambs (indicated by asterisks) and control lambs for 1982 and 1983 (Ram Mountain).

1982

ID	LPG	DATE OF COLLECTION
blk-yel	0	10 June
*blk-org	0	10 "
*org-blk	0	10 "
blu-blu	21	10 "
org-blu	0	10 "
org-wht	0	10 "
blu-yel	0	13 "
yel-yel	18	13 "
yel-wht	0	13 "
blu-org	0	13 "
*org-org	20	19 "
*org-org	0	29 "
*blk-wht	2	29 "
wht-org	134	7 July
blu-yel	7	7 "
blu-wht	211	7 "
blu-wht	14	12 "
yel-blu	169	22 "
yel-blk	3	22 "
blk-blu	33	25 "
wht-blk	41	4 August
yel-yel	30	4 "
*org-blk	221	4 "
blu-wht	5	4 "
blu-blu	4	4 "
wht-wht	40	5 "
org-wht	46	5 "
blu-org	84	5 "
*blk-org	465	6 "
yel-wht	130	6 "
wht-org	160	6 "
*blk-wht	251	13 "
blk-yel	874	17 "
*blk-org	1090	18 "
yel-org	10	21 "
*org-yel	644	21 "
*blk-wht	34	21 "
yel-blk	21	24 "
blu-wht	182	24 "
blu-yel	112	27 "
yel-blu	173	29 "
wht-yel	253	30 "
blu-blu	56	30 "
org-org-	15	15 September
org-wht	233	15 "
yel-yel	275	15 "
blk-yel	468	15 "
*blk-org	651	15 "
*blk-wht	47	15 "

ID	LPG	DATE
yel-wht	71	17 September
blu-wht	72	20 "
* blk-wht	150	20 "
wht-yel	492	20 "
org-wht	344	22 "
* org-yel	1102	22 "
blu-blu	243	22 "
yel-blu	368	23 "
blk-blu	93	23 "
org-org-	271	1 November
org-wht	380	1 "
yel-blu	251	23 "
blu-yel	478	23 "
wht-wht	570	23 "
* org-blk	800	23 "
* org-org	693	23 "

1983

ID	LPG	DATE OF COLLECTION
* wht-wht	0	17 June
* blu-blu	0	17 "
* yel-yel	0	22 "
* wht-wht	400	23 July
* blu-blu	356	23 "
un 1	346	23 "
un 1	280	23 "
un 1	140	23 "
* yel-yel	3288	23 "
un 1	279	24 "
un 1	233	24 "
org-org	293	24 "
un 1	15	26 "
* blu-blu	24	26 "
* yel-yel	1368	26 "
un 1	211	29 "
* wht-wht	1004	29 "
* yel-yel	3514	29 "
wht-yel	208	2 August
* wht-wht	250	2 "
* wht-wht	386	2 "
org-org	480	4 "
un 1	405	4 "
* yel-yel	3108	4 "
un 1	28	4 "
un 1	43	4 "
un 1	25	5 "
* blu-blu	2774	6 "
* yel-yel	3707	6 "
un 1	6	7 "
un 1	0	7 "
* blu-blu	1982	7 "
* wht-wht	1331	7 "
un 1	132	7 "
blk-org	450	8 "
* yel-yel	1050	8 "
org-blk	7	8 "
blk-blk	266	8 "
blu-org	560	8 "
org-wht	36	11 "
blk-wht	259	11 "
* wht-wht	707	12 "
blk-blk	426	12 "
* blu-blu	329	12 "
wht-org	4	18 "
org-yel	44	19 "
yel-wht	172	19 "
* blu-blu	34	19 "
un 1	180	22 "

ID	LPG	DATE
* wht-wht	348	23 August
blu-blk	0	23 "
blk-blu	313	23 "
org-wht	16	28 "
blk-wht	116	28 "
yel-blk	497	28 "
yel-org	154	28 "
* blu-blu	147	29 "
* yel-yel	809	29 "
blk-blk	977	29 "
* wht-wht	5402	7 September
* blu-blu	1828	13 "
* yel-yel	3180	13 "
org-org	354	13 "
blk-blk	468	13 "
blu-org	236	13 "
un 1	206	13 "
* yel-yel	777	15 "
* blu-blu	10	16 "
yel-yel	227	16 "
blk- blu	410	16 "
wht-blk	90	16 "
blu-wht	55	16 "
yel-blk	44	16 "